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MEMBRANES FOR ULTRAFILTRATION, OF GRADUATED FINENESS DOWN TO MOLECULAR SIEVES.

By J. W. McBAIN and S. S. KISTLER.*

(From the Chemical Laboratories of Stanford University, Stanford University, California.)

(Accepted for publication, May 29, 1928.)

It is becoming increasingly evident that ultrafiltration is amongst the most important methods of colloidal and biological investigation. In many instances ultrafiltration is the chief test for the presence or existence of substances in the colloidal state. In medicine it is becoming an indispensable tool in the study and control of filter-passing viruses and in physical chemistry new fields of investigation of the constitution of aqueous and non-aqueous solutions open out with the advent of graded molecular sieves. For all these reasons it is desirable that trustworthy membranes should be universally familiar.[1]

For many years one of us has advocated the use of sheets of cellophane for use in ultrafiltration. They possess the advantage of being obtainable commercially in large sheets or batches which are approximately uniform in their properties.[2] The material of which they are composed is as non-reactive as filter paper consisting of cellulose with a trace of glycerol.[3] Cellophane may be obtained in various forms but

* With addenda by M. C. Field.

[1] An example of the many almost unexplored possibilities of the quantitative use of ultrafiltration may be seen in a previous paper by McBain and Jenkins (McBain, J. W., and Jenkins, W. J., *J. Chem. Soc.*, 1922, cxxi, 2325) where it is applied to the study of soap solutions measuring not only amounts of colloid and crystalloid present but also hydrolysis, osmotic pressure, hydration or solvation of the material held back by the ultrafilter and even relative proportions of the different kinds of colloidal particles.

[2] For example, they are now manufactured by the E. I. du Pont de Nemours and Company.

[3] In filtering soap solutions through collodion membranes obtained from collodion dissolved in glacial acetic acid we found that there was an appreciable proportion of cellulose acetate in the membrane which entered into double decomposition with the soap and distorted certain of the quantitative measurings.

the flat sheets are especially convenient for clamping in ultrafilters which may be operated under high pressures[4] up to 100 atmospheres. Cellophane is much stronger than collodion whilst the latter, under high pressures, tends to become embedded in the very fine bronze gauze which forms the most suitable support and therefore becomes difficult to replace by a fresh membrane. Cellophane which has been swollen

TABLE I.

Rates of Filtration of Various Liquids Through "600" Cellophane.

Solution	Pressure used kg./sq. cm.	Filtrate per sq. cm. per hr. per kg. per sq. cm.
Water	47	0.02
$AgNO_3$ in piperidine	17	0.0009
NH_4I in aniline	15	0.00004
NaBr in C_2H_5OH	75	0.0001
H_2O in paraldehyde	50	None
H_2O in amyl alcohol (iso)	50	None
$AgNO_3$ in $(C_2H_5)_3NH$	14	None

in water possesses pores so large that no molecules, but only colloidal particles are held back.

Cellophane is obtainable in several different degrees of porosity as measured for example by the bubble test[5] and it will be shown that there are several methods of progressively enhancing the fineness of

[4] The most convenient form of ultrafilter which we have used is that supplied by Vereinigung Göttinger Werke, Göttingen (with or without electrical stirrer) which is attached directly to a cylinder of nitrogen and will stand pressures up to 150 kilos per sq. cm.

[5] See Bechhold, H., *Z. phys. Chem.*, 1907, lx, 257; also 1908, lxiv, 330, in both of which papers he made a mistake of a decimal point expressing his pore diameters as was pointed out by Bigelow, S. L., and Bartell, F. E., *J. Am. Chem. Soc.*, 1909, xxxi, 1197, and implicitly accepted by Bechhold in his later papers (see Bechhold, H., and Szidon, V., *Kolloid-Z.*, 1925, xxxvi (Ergänzungsband Zsigmondy) 264). The results of the method are not entirely free from contradictions although it is the best available and certainly yields comparative results. It measures only the few largest pores, and much of the filtration must occur through smaller ones. It seems as if a Bechhold ultrafilter holds back particles whose diameter is only about one-fifth of the size of the largest pores.

these membranes until they hold back first the larger crystalloidal molecules and then smaller ones, until the smallest molecules and ions are retained. For example, it is easy to separate by ultrafiltration sucrose from methyl alcohol in aqueous solution. Lastly, it will be shown that these membranes can be used in the study of non-aqueous solutions with such solvents as benzene, amyl alcohol or paraldehyde.

The Permeability and Swelling of Cellophane Membranes in Various Solvents.

Water passes through ordinary cellophane very readily. Piperidine passes through slowly but at a satisfactory rate for ultrafiltration,

TABLE II.

Swelling Produced by Soaking Cellophane "600" in Various Solvents.

Solution	Initial thickness	Thickness in solution after lapse of stated time			
	mm. = 0.002	3 hrs.	15 hrs.	24 hrs.	150 hrs.
	mm.	mm.	mm.	mm.	mm.
Water	0.047	0.098	0.098		
95 per cent C_2H_5OH	0.045	0.047	0.047	0.048	0.048
Amyl alcohol	0.045	0.044	0.043	0.044	0.044
Paraldehyde in water (saturated solution)	0.047	0.090	0.093	0.093	0.093
Aniline	0.040		0.042	0.041	0.042

while ethyl alcohol and aniline filter only extremely slowly. Amyl alcohol, paraldehyde and diethylamine pass too slowly to be measured in a reasonable length of time.

Table I gives the rates of filtration for solutions using these solvents. The cellophane used was "600," one of the densest obtainable commercially.

Since Brown (1) has found that the rate of diffusion of substances through a collodion membrane, and also the apparent reticular size, or the porosity, depends directly upon the previous swelling of the membrane it seemed possible that different degrees of swelling of the cellophane produced by contact with the solvent could explain the wide differences in permeability for the different solvents in Table I.

Accordingly swelling experiments were undertaken, the results of which are shown in Table II. It was found that the sheets of cellophane expand to different extents in different directions. Thus whereas it is shown in Table II that the thickness is increased by over 100 per cent by soaking in water the sheet does not expand at all in length, and expands only 9 per cent in width. It is a great advantage that the area of the cellophane is so little affected. The difference in expansion in the two directions is presumably brought about by the squirting through the slit in the factory and might be interpreted as evidence of an incipient fibrous structure. It is seen from Table II that there is a rough correlation between the amount of swelling induced by the solvent and the permeability of the membrane for that solvent.

It was attempted to increase the permeability of the cellophane to non-swelling liquids by previously swelling the membranes in water and then attempting to force the particular solution through. To our surprise, this was of no avail except in the cases of ethyl alcohol, diethylamine and ethylamine. The membranes remained as impermeable to aniline, amyl alcohol and paraldehyde as ever.

In order to keep the membrane in as nearly the same thermodynamic environment as possible throughout the experiment, new trials were made, this time filtering two phase solutions instead of one. For example, water and amyl alcohol were shaken together till both the aqueous and alcoholic layers were thoroughly in equilibrium with each other. In each of the three cases tried the aqueous layer was the denser so that it came into contact with the membrane first. The membrane was swollen in water first before being placed in the ultrafilter. The aqueous layer passed through the membrane rapidly with apparently no change in concentration, with the possible exception of the aniline solution in which it seemed as though some aniline were being retained (analysis unsatisfactory).

As soon as the upper (non-aqueous) phase reached the membrane, filtration ceased. This was to be expected, for it is exactly like the bubble test of Bechhold.[5] Frequently when a liquid has all gone through a dense ultrafilter the wet membrane will completely hold back the compressed nitrogen. It requires a pressure of 100 atmospheres to blow a bubble through a hole 30Å. u. (30×10^{-8} cm.) in diameter if the hole is covered with water. The pressure is inversely

proportional to the diameter of the opening and directly proportional to the surface tension of the liquid. If there are two liquids as in the present case the interfacial tension plays the same rôle as the surface tension of one liquid against nitrogen or air, and if all the holes are smaller than the calculated value the meniscus cannot pass through.

According to this explanation, a membrane saturated with one liquid should be permeable to another liquid that is completely misci-

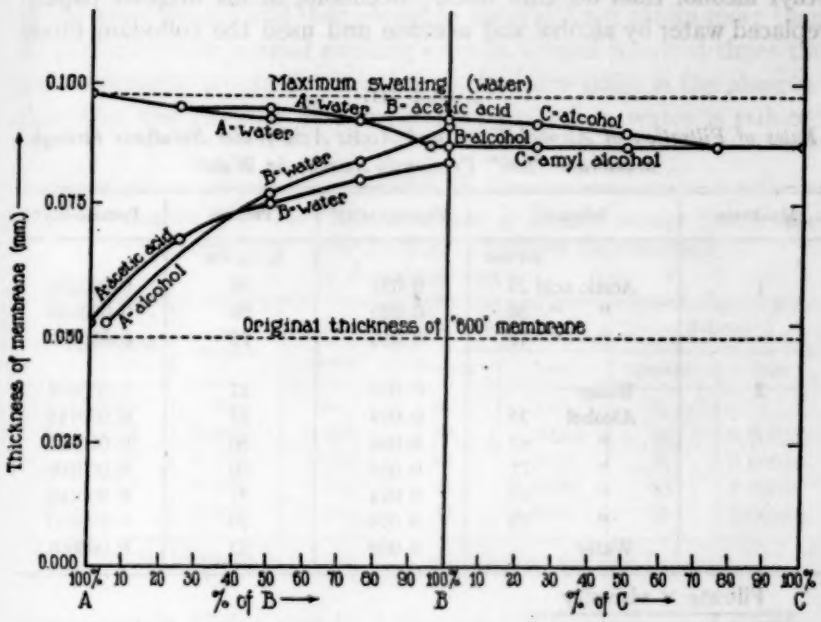

FIG. 1. Thickness of "600" cellophane when placed successively in a solvent *A*, then in mixtures in which *A* is replaced by a second solvent *B*, then in some cases by a third solvent *C*.

ble with the first. Our observations on the permeability of cellophane, saturated with water, to alcohol and the amines support this view. Since the cellophane becomes permeable to alcohol and the amines by swelling in water; that is, a liquid with which they are miscible, it is possible to make the membrane permeable to the other liquids by using an intermediary liquid such as alcohol with which they in their turn are miscible. Thus a membrane, swollen in water and soaked

successively in 25, 50, 75 and 95 per cent ethyl alcohol and then in several mixtures up to amyl alcohol, proved readily permeable to the latter as well as to benzene, aniline and paraldehyde. It is just as effective to transfer the cellophane directly from water to ethyl alcohol and then to the other liquid except in the case of paraldehyde. Here the final permeability was produced much more quickly by proceeding in stages. Thick membranes always require longer soaking in the ethyl alcohol than do thin ones. Bechhold, in his original paper,[5] replaced water by alcohol and acetone and used the collodion filters

TABLE III.

Rates of Filtration of Alcohol-Water and Acetic Acid-Water Solutions through Membrane "600" Previously Swollen in Water.

Membrane	Solution		Viscosity at 25°	Pressure	Permeability*
		per cent		*kg./sq. cm.*	
1	Acetic acid	25	0.024	50	0.000206
	" "	50	0.020	50	0.000103
	" "	75	0.016	75	0.000079
2	Water		0.009	47	0.000097
	Alcohol	25	0.019	53	0.000115
	"	47.5	0.024	50	0.000120
	"	75	0.019	50	0.000100
	"	95	0.014	51	0.000105
	"	75	0.019	50	0.000103
	Water		0.009	53	0.000091

* $\frac{\text{Filtrate} \times \text{viscosity}}{\text{Time} \times \text{pressure} \times \text{area}}$.

for ultrafiltration of alcoholic chlorophyl. In his paper in 1925[5] he obtained membranes for use with non-aqueous solvents by coagulating collodion with such solvents, the best instance being collodion dissolved in ether-alcohol for impregnation of filter paper, coagulated by toluene.

Fig. 1 shows the changes in thickness of the membrane which accompany such treatment with various solvents. Clearly, the solvent causing the swelling is water. Neither alcohol nor glacial acetic acid produces appreciable swelling. Once swollen, the cellophane

retains most of its thickness when the water is replaced by another solvent. The slight but distinct shrinkage on replacing water by ethyl alcohol is the same whether the transition is made in stages or directly. The membrane did not reach its full swelling when it was placed first in acetic acid or alcohol and then in water.

In the case of isoamyl alcohol, there seems to be a more pronounced inhibiting effect on the swelling. A membrane that had been soaked in the amyl alcohol and then placed in water did not attain more than 80 per cent of its normal swelling even in several hundred times the time ordinarily required. Bearing on the same point is the observation that the swelling of a membrane soaked in water is reduced

TABLE IV.

Rates of Filtration of 95 Per Cent Alcohol and of Benzene through Specimens of Cellophane "600" (after Swelling in Different Concentrations of Aqueous Alcohol).

Swelling solution	Initial thickness	Thickness in 95 per cent alcohol	Alcohol		Benzene	
			Pressure	Rate*	Pressure	Rate
per cent	*mm.*	*mm.*	*kg.*		*kg.*	
Alcohol 75	0.060	0.058	38	0.00088	36	0.00018
" 50	0.075	0.070	37	0.0026	31	0.00034
" 25	0.082	0.075	31	0.0039	35	0.00039
Water	0.095	0.082	37	0.0037	38	0.0013

* Cc. divided by pressure (kg./sq. cm.), time (hours) and area (sq. cm.).

approximately 35 per cent by transferring it to a saturated solution of amyl alcohol in water, whereas if the membrane is first transferred to 95 per cent ethyl alcohol from the water and then placed in the amyl alcohol, the swelling is reduced only about 20 per cent.

The results in Table III show that swelling does not alone determine the permeability of the membrane. The last column is the value for the amount of filtrate multiplied by viscosity and divided by time, pressure and area.

On the other hand Table IV shows that swelling has some association with permeability. Different pieces of cellophane showed a permeability to alcohol and thereafter to benzene that increased rapidly with amount of the initial swelling.

Cellophane that is thoroughly washed and afterwards dried at 100° is hard and brittle, the same is true if the drying is conducted over sulfuric acid at room temperature. A membrane that is washed thoroughly with water and then placed in 95 per cent alcohol and then in amyl alcohol and allowed to dry at room temperature was not brittle but was completely impermeable to amyl alcohol. Another similar membrane transferred from the amyl alcohol to benzene before it was dried was impermeable to benzene. This is a great contrast to the experiments described in connection with Fig. 1 where the membrane is not dried.

Preparation of Dense Ultrafilters.

Cellophane swollen in water allows all such undoubted simple molecules as sucrose to pass through freely so that the filtrate has the same composition as the original liquid. However it does hold back the larger colloidal particles. Cadmium iodide dissolved in amyl alcohol is probably an example of a non-aqueous colloidal electrolyte, judging by its migration number. Upon filtering it through swollen cellophane the solution above the membrane became distinctly more concentrated to the extent of 4 per cent. A membrane originally swollen in 50 per cent ethyl alcohol was evidently somewhat denser, for the solution of cadmium iodide above the membrane was increased in concentration to 144 per cent of its original value. For a systematic investigation of this field of physical chemistry in aqueous and non-aqueous solutions denser membranes had to be developed. Bechhold was unsuccessful in his attempts to make such ultrafilters but several authors have described very dense membranes which however they have not used for actual ultrafiltration (see for example Northrop, J. H., *J. Gen. Physiol.*, 1928, xi, 233; and Michaelis, L., Colloid Symposium Monograph, 1928, v, 135). It must be strongly emphasized that sieve action or ultrafiltration bears no relation to thermodynamic calculations based upon equilibrium or to osmosis anomalous or otherwise, or to diffusion or to electroosmosis. This distinction is almost invariably lost sight of.

Clogging of the membrane may take place when filtering a solution, and it occurred to us that it might be possible to control the permeability of a membrane by purposely clogging it with some insoluble

substance.[6] A highly dispersed colloid would seem to be the best suited for the purpose. Accordingly solutions of cellulose in Schweitzer's reagent were filtered through the cellophane, cellulose being preferred because of its insolubility in the liquids under investigation. A small quantity of the cellulose solution diluted with water and filtered through the membranes greatly reduced their permeability. The reduction in permeability can be fairly well controlled reasoning from our few experiments. Moreover the rate of filtration remains satisfactory even when molecules such as sucrose are being held back.

A troublesome difficulty met with was the tendency of the bronze gauze backing for the membrane in the ultrafilter to dissolve in the ammoniacal solution. Gold plating the gauze proved to be a protection for several filtrations, but eventually failed. As a consequence, the stock solution of cellulose had to be diluted by 5 or 10 times its original volume with water before being used. The stock solution was made by dissolving 5 gm. of C.P. copper carbonate in 100 ml. of concentrated ammonium hydroxide and saturating with cellulose.

The permeability of the membrane decreased very rapidly with the first small quantity of cellulose filtered and continued to decrease with more solution but at a slower and slower rate apparently approaching a minimum when relatively small amounts of cellulose had been deposited. Probably the rapid decrease at first can be accounted for by the clogging of the larger openings in the reticular structure. The probabilities are that the imbedded membrane produced by filtering a colloid out of solution is much more uniform than the untreated cellophane. When the larger openings have been closed, the additional decrease in permeability effected by further depositing the cellulose would come mainly from the added friction to the passage of the solution and from the reduction of all of the openings to the average size of the superimposed layer.

Observations on films of collodion have led us to believe that more

[6] McBain and Jenkins[1] used such clogging of the pores as evidence that the so called emulsoids are not emulsions because true emulsions do not clog the pores. Bigelow and Bartell[5] have clogged pores of porcelain with barium sulfate and sulfur but that was on a much coarser scale. Much earlier Martin (Martin, C. J., *J. Physiol.*, 1896, xx, 364) prepared ultrafilters by impregnating the wall of a Pasteur-Chamberlain filter with gelatin or silicic acid.

concentrated solutions of cellulose produce much smaller limiting permeability. Another method of decreasing the permeability is by filtering solutions of collodion in alcohol-ether through a membrane that has been previously swollen in water and then transferred to alcohol. The result is to deposit and embed upon the upper surface of the cellophane an exceedingly thin film of collodion. The range of permeabilities extends down to the completely impermeable condition.

The larger the percentage of alcohol in the collodion, the greater the permeability of the treated cellophane as measured by the rate of filtration in pure water. There seems to be a compacting of the membrane after the solvent has filtered through if the pressure is left on for several hours. The densest membranes that we have made have been made with collodion in 50 per cent ether in alcohol solvent and allowed to compact after the solvent has passed through. Such a membrane even when the collodion is not over 0.007 mm. thick is practically completely impermeable to water. A membrane of this kind will pass a strong alcohol solution, and thereafter it proves to be more permeable to water, the permeability depending upon the percentage of alcohol in the solution that was filtered. If an alcohol-water solution is allowed to stand over a collodion membrane, made as above, without pressure for an hour or two and then replaced with water it is found to have had a much greater effect in increasing the permeability. It seems quite evident from this fact that the collodion in the alcoholic solution is soft enough to be compacted by the pressure. Replacing alcohol solution with water probably hardens the collodion enough to make it resist deformation.

The remaining observations in this paper were made by M. C. Field at Bristol University, Mr. W. F. K. Wynne-Jones having previously shown that sucrose solutions passed unchanged through the densest membranes formed by the deposition of nickel on phosphor bronze as described by Manning, *J. Chem. Soc.*, 1926, 1327. Mr. Field found that the densest membranes of cellophane and membranes of cellulose acetate likewise had no effect, behaving in this respect like filter paper or bacteriological filters.

Collodion Membranes.

The general method used in their preparation was as follows: 9 cc. of 14 per cent collodion in 50/50 ether-alcohol as specially sup-

plied through the kindness of the British Xylonite Company were diluted by the addition of a further 27 cc. of 50/50 ether-alcohol, making a total of 36 cc. of 3.5 per cent collodion in ether-alcohol. This is then poured into the lower half of a desiccator (diameter 4½ to 5 inches) containing clean mercury, and allowed to stand for several hours until set. When sufficient solvent has evaporated, the membrane is removed by cutting round the edges with a penknife and lifting from the mercury surface. It is then placed between filter papers to shrink for a definite time, the final permeability depending to a large extent on the time which elapses during this period of shrinking.

The membrane is finally placed under water for 24 hours and is then ready for use. The method used to change the permeability, which had been employed up to the present, depends on the fact that the permeability increases when the membrane is soaked in alcohol-water mixtures. Therefore, by allowing the membrane to shrink until it is quite impermeable to even water molecules and then soaking it in a definite alcohol-water mixture, a standard type of membrane can be obtained (see (1) pages 40, 591). This method has been used by Mr. Field. The higher the percentage of alcohol in the alcohol-water mixtures the more permeable is the resulting membrane. Also the variation in permeability is very much greater for high percentage alcohol mixtures than for low ones, *i.e.* alcohols of say 10 to 60 per cent do not differ in their action on the membrane so greatly as alcohols of from 90 to 95 per cent.

Thus the apparent pore size of any undried collodion membrane depends only on the per cent of alcohol in the solution in which it is soaked. That is to say, variation in thickness of the membrane in the time taken to make and in the time taken to dry, or in the per cent collodion in the original solution, makes no difference to the proportions of the constituents in a solution held back by the membrane, so long as it is soaked for a sufficient length of time in the alcohol solution. Two such similar membranes will let through a filtrate of the same composition provided that both are allowed enough time, even where the time required for the actual filtration differs many fold.

For all practical purposes, the effect of soaking in alcohol is completed in 12 hours. After that length of time only inappreciable alterations in the permeability take place.

In the later work, using these collodion membranes a somewhat different method of preparation was used. 60 cc. of 3.5 per cent collodion in 50/50 ether-alcohol were poured into a fairly shallow dish on to a surface of mercury (7½ inches in diameter) and allowed to set, when it shrinks from the sides of the vessel, to which it adheres for some time after it has set, it is removed from the surface and placed between blotting paper until completely dried out. The permeability is then adjusted by the per cent alcohol in an alcohol-water mixture in which it is soaked preparatory to placing in the ultrafilter.

Brown's method (1) for preparing a 10 per cent shrinking membrane by pouring 8 per cent collodion in 50/50 ether-alcohol over a clean glass plate and setting by immersion in water, was repeatedly tried with little success. The membranes invariably contained bubbles which made them quite unsuitable for high pressure ultrafiltration work.

One thick collodion membrane was obtained after soaking in 60 per cent alcohol which held back sucrose in aqueous solution giving almost pure water as filtrate and showing no clogging of the pores with time.

Attempts were next made to repeat this result with a second membrane prepared in exactly the same manner. This, however, could not be done; a large number of membranes were prepared but their chief feature was one of variability. Thus, sometimes the above semipermeability was obtained and in other cases, the membrane was either completely impermeable or completely permeable to both sugar and water. All states between these limits were obtained.

A membrane which under 100 atmospheres pressure allowed 2 cc. of water to come through from N/2 sucrose for 24 hours allowed only 3 drops of 25 per cent aqueous alcohol to pass through in a week, although the aqueous alcohol was unchanged. Such membranes, holding back large proportions of sucrose, likewise hold back large proportions of potassium chloride, which may even crystallize out in solid form as the residue becomes concentrated. However, from a quantitative standpoint no two results are obtained alike and the collodion membranes appear to alter whether they are allowed to dry and then resoaked in alcohol, or whether they are kept beneath water, or in their standard alcohol-water mixture.

Animal Membranes—Pig's Bladder.

Gold beater's skin (see Bigelow and Gemberling (3)), Bedicher (a membrane obtained from the cow's intestine) and pig's bladder membranes were each experimented with. The latter are the only ones to give any satisfaction as molecular sieves although Bedicher holds back a slight amount of sucrose. The Bedicher is very fine, like tissue paper, and filters very rapidly and should be very useful with fine colloids. The membranes were prepared by inflating the pig's bladder in the usual manner and allowing it to dry until brittle. Different types of membranes were obtained according to the extent of inflation. The greater the tension the greater the porosity. Once obtained their properties remain practically constant. These membranes filter very much more rapidly than collodion.

Pig's bladder holds back only $\frac{1}{2}$ to $\frac{3}{4}$ of the sugar in aqueous sucrose. It also holds back a portion of potassium chloride, and to a less extent glycerol and urethane from their aqueous solutions. In each case of course the residue becomes more concentrated. The filtrate from aqueous ethyl and methyl alcohol is likewise somewhat diluted.

Bechhold (Bechhold, H., *Z. phys. Chem.*, 1907, lx, 262) tested parchment paper, fish bladder and amnion membrane and found the fish bladder most uniform though not as uniform as his artificial membrane.

SUMMARY.

The use of cellophane in ultrafiltration is recommended. It is shown that after it has been swollen in water it does not hold back molecules such as sucrose but that it holds back all but the finest colloidal particles. Two methods are given for progressively decreasing the size of the pores until the cellophane becomes a very fine molecular sieve. A sieve structure as the chief factor seems most in accordance with our experience of this and other ultrafilters. Collodion membranes may also be used as molecular sieves but their properties are inconstant. Bedicher is a very fine and rapid filtering ultrafilter and pig's bladder holds back a fair proportion of such molecules as sucrose and potassium chloride. Notes are made on the behavior of cellophane in aqueous and non-aqueous solutions. It is emphasized that ultrafiltration is distinctive and has but little relation to diffusion, dialysis, osmosis, electroosmosis or thermodynamics.

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EFFECTS OF CYANIDE ON THE PROTOPLASM OF AMEBA.

By FLOYD J. BRINLEY.*

(From the Zoological Laboratory of the University of Pennsylvania, Philadelphia.)

(Accepted for publication, May 30, 1928.)

Within the last 75 years a vast amount of information has been obtained concerning the physiological action of cyanide on organisms. Most of the literature, however, deals primarily with the effects of potassium cyanide on oxidation, while comparatively little information seems to exist in regard to the direct action of cyanide on protoplasm. Since most of our information was obtained from oxidation experiments with potassium cyanide (1, 2, 3), and since it is known that hydrogen cyanide passes through living membranes (4, 5), it was thought desirable to study in some detail the effects of hydrogen cyanide and potassium cyanide on the physico-chemical properties of protoplasm. The work here reported deals with the effects of hydrogen cyanide and potassium cyanide on the viscosity of the protoplasm of a small species of ameba of the *proteus* group.

Method.

Amebæ were immersed in aqueous solutions of HCN or KCN and the same solutions were injected into other individuals by means of Chambers' micromanipulator. The concentrations of the cyanide solutions ranged from N/10 to N/3,000 and the pH values were changed by the addition of NaOH or HCl. The organisms were studied with transmitted light and with dark-field illumination. It was previously determined that Brownian movement (6) may be used as an index of the comparative viscosity of the protoplasm of amebæ; an increase in the rate of Brownian movement denotes a decrease in the viscosity and a decrease in the movement an increase in viscosity. One criticism of this method is the lack of quantitative results. It has, however, an advantage over other methods in that the viscosity changes in the protoplasm may be noted without interfering with the organism under observation.

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* National Research Fellow.

Immersion Experiments.

N/10 HCN: Amebæ immersed in N/10 HCN solutions, with pH values of 4.6, 7.0 and 7.4, show an immediate increase in protoplasmic viscosity which is quickly followed by an internal reorganization of the protoplasm. The granular portion of the protoplasm collects into numerous minute spheres which are suspended in a hyaline matrix (Fig. 1). The spheres are gelled at pH 4.6 while at pH 7.0 and 7.4 the granules within the spheres show slight Brownian movement indicating a gel of low viscosity. The separation of the granular portion of the protoplasm into dense spheres also occurs when amebæ are immersed in a HCl solution with a pH of 4.6. Higher concentrations of HCl (pH 1.6) produce an immediate gelation of the entire protoplasm (6). Thus it appears that the formation of the granular spheres is an intermediate stage between liquefaction, which occurs when amebæ are exposed to dilute cyanide solutions, and gelation of the protoplasm.

N/100 HCN: When amebæ are immersed in N/100 HCN solutions with pH values 5.0, 6.6 or 7.4, the viscosity of the protoplasm is slightly increased for a few seconds, then followed by a swelling of the protoplasm and a marked increase in the rate of Brownian movement. The cell assumes a spherical form and the contractile vacuole enlarges enormously and finally breaks, liberating the enclosed fluid which mixes immediately with the cytoplasm. The cell membrane is either ruptured, allowing the liquid cytoplasm to escape into the surrounding medium or it remains intact. If the cell membrane is not broken, the granular portion of the protoplasm collects into one large sphere which is suspended in a hyaline medium (Fig. 2). The cell membrane eventually dissolves and the cell disintegrates.

N/300 HCN: Amebæ placed in N/300 HCN solutions with pH values of 5.6, 6.6 and 7.4, remain active for a few seconds, then locomotion ceases and the animal retains its life-like position with its pseudopodia extended. This quiescent stage is accompanied by an increase in viscosity of the protoplasm and the animals ultimately swell and assume a spherical form, which is characterized by a marked decrease in viscosity. The contractile vacuole enlarges to many times its original size and several accessory vacuoles appear. The surface

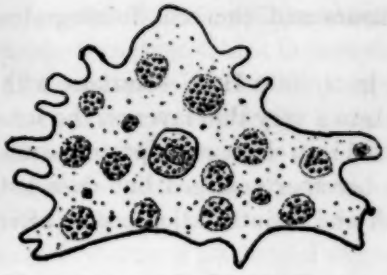

FIG. 1.

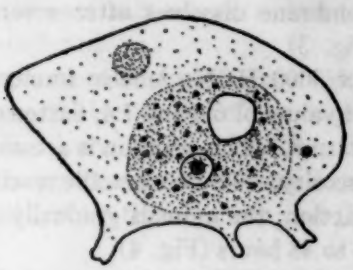

FIG. 2.

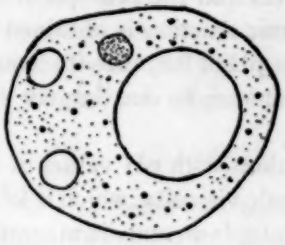

FIG. 3.

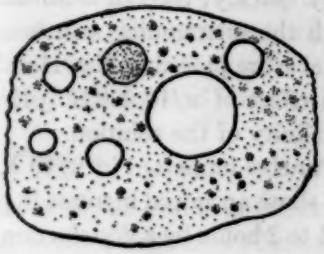

FIG. 4.

FIG. 5.

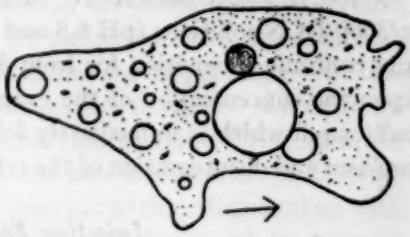

FIG. 6.

FIG. 1. Free-hand sketch of ameba killed with N/10 HCN, showing the formation of minute spheres of protoplasm suspended in a hyaline area.

FIG. 2. Sketch of ameba killed in N/100 HCN. Protoplasm collected in one large sphere.

FIG. 3. Sketch of ameba immersed in N/300 HCN, showing the enlarged contractile vacuole.

FIG. 4. Drawing of ameba immersed in N/3000 HCN for 16 hours.

FIG. 5. Drawing of ameba immersed in N/100 KCN, showing the limax form.

FIG. 6. Sketch of ameba injected with HCN or KCN. Animal alive and moving in the direction indicated by the arrow.

membrane dissolves after several hours and the cell disintegrates (Fig. 3).

N/3000 HCN: Amebæ immersed in N/3000 HCN solutions with pH values of 6.6 and 7.4, flatten out into a very thin layer on the substratum. This reaction is accompanied by a decrease in protoplasmic viscosity. The contractile vacuole becomes enlarged but does not function; the animals gradually swell and disintegration occurs after 24 to 48 hours (Fig. 4).

N/10 KCN: Amebæ immersed in N/10 KCN solution, pH 9.8, die very quickly; the cell membrane dissolves and the protoplasm mixes with the surrounding solution. The same reaction is obtained when amebæ are placed in NaOH or KOH at a pH of 9.8, thus it seems that the effect of N/10 KCN (pH 9.8) on amebæ is due largely to the alkalinity of the solution.

Amebæ immersed in N/10 KCN solutions with pH values of 6.8 or 7.4 show an initial increase in protoplasmic viscosity, which is followed in 1 to 2 hours by a liquefaction of the protoplasm and disintegration of the cell.

N/100, N/300, N/3000 KCN: Amebæ immersed in N/100, N/300 and N/3000 KCN solutions (pH 6.8 and 7.4) elongate into the limax form and continue locomotion for from 30 minutes to 24 hours depending upon the concentration of the cyanide solution. The animals gradually swell which is immediately followed by liquefaction of the protoplasm and disintegration of the cell occurs within 3 days (Fig. 5).

Injection Experiments.

By means of the Chambers' micromanipulator, amebæ were injected with HCN or KCN. The concentrations ranged from N/10 to N/3000 and the pH values from 4.6 to 7.4. The cyanides at different concentrations and with different pH values produce similar effects on the internal protoplasm of amebæ. The animals after injection become highly vacuolated and assume a fan shape (Fig. 6). The anterior end spreads out into a very thin layer on the cover slip. Brownian movement and streaming of the protoplasm is accelerated; the contractile vacuole enlarges slightly but usually does not contract unless it is lodged in an angle of an advancing pseudopodium or comes

in contact with the cell membrane. The animals recover unless the dosage is so large that it injures the membrane.

Insertion of a needle into the protoplasm of an ameba results in a local gelation at the point of stimulation. As locomotion of the animal continues the gelled area lags behind and eventually constitutes the posterior region of the organism and the ameba assumes a limax form. Ultimately the gelled region is assimilated by the organism and the animal returns to its normal shape.

Injections of distilled water into amebæ result in a dilution of the protoplasm, streaming of the protoplasm is increased and the contractile vacuole contracts at irregular intervals. For a more detailed account of the effect of injections of distilled water on the contractile vacuole the reader is referred to the paper of Howland and Pollack (7).

Tearing the Surface Membrane.

The cell membrane of a normal ameba is elastic and it is possible to stretch it by means of microdissection needles into a long thread-like structure which appears to be fibrous in texture. When released the membrane returns to approximately its former position. With considerable care the animal may be pulled into two parts without any escape of the internal protoplasm and the two sections will continue locomotion. When a small tear is made in the cell membrane, a portion of the internal protoplasm flows from the torn surface and the injury is repaired by the formation of a new membrane. If the tear is sufficiently large, the protoplasm flows out of the cell and mixes with the surrounding water. When amebæ are immersed in aqueous solutions of HCN or KCN and the cell membrane torn, a new membrane is formed over the injured surface, which indicates that the internal protoplasm has not been greatly injured.

Injection Followed by Immersion.

The results of the experiments on immersion, injection and tearing of the cell membrane, seem to indicate that the toxicity of cyanides is due to their action on the cell membrane and that the internal protoplasm is not permanently injured. Since it has been shown that HCN (4, 5) passes through living membranes and that the cyanides decrease

the viscosity of the internal protoplasm of amebæ, it was thought desirable to ascertain whether the toxicity of cyanide is due entirely to its effect on the the cell membrane or to an effect on the membrane and the internal protoplasm. Twenty-five to thirty amebæ in a single experiment were injected with HCN or KCN and then quickly immersed in cyanide solutions. A higher concentration of intracellular cyanide was obtained by this means than would have been obtained from immersion in a cyanide solution. If the toxicity of cyanide is due to an effect on the cell membrane and internal protoplasm, the injected individuals should die in a much shorter time after immersion than the uninjected animals. The results, however, show that the injected organisms do not die any sooner after immersion, than do the uninjected cells immersed in the same cyanide solutions. This seems to indicate that the toxic action of cyanide is exclusively a surface one.

SUMMARY.

The experiments seem to indicate that the toxicity of HCN and KCN for amebæ is due to their effect on the cell membrane and not on the internal protoplasm.

Concentrated solutions (N/10–N/300) of HCN or KCN produce an initial increase in viscosity of the protoplasm of amebæ (immersed) which is followed by liquefaction and disintegration of the cell. Dilute solutions of HCN or KCN decrease the viscosity of the protoplasm of amebæ.

Injections of HCN or KCN into amebæ produce a reversible decrease in viscosity of the protoplasm.

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THE INJECTION OF SULFATES INTO VALONIA.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, May 24, 1928.)

Cells of *Valonia macrophysa*, while ordinarily free from sulfate in the vacuolar sap,[1] occasionally are found in apparently normal state yet giving a test for this ion. In order to determine its effect directly, the following experiments were performed in Bermuda in 1924. The number of cells was small (30) but the results sufficiently definite to justify certain conclusions. The method furthermore is of interest in view of its later employment for the injection of toxic substances.[2]

Two successive operations were employed. A small amount of sap (about 1/8 of the cell volume) was withdrawn through a fine capillary. This was removed and a second capillary inserted, containing the solution for injection. The contents were forced in under air pressure (taking care that no bubbles entered) until the cell was turgid. The capillary was removed while the pressure was still being applied.

Mortality was high due to the two punctures and the consequent temporary softness of the cell, but a small number of cells survived in both of two groups. In group A a solution of 0.6 M KCl was injected. Of these 2 cells survived and lived 2 weeks, when the experiment was discontinued. In group B a solution of 0.4 M K_2SO_4 was injected, and 3 cells survived. At the end of 2 weeks the sap was removed from all the cells and tested for sulfate ion. Group A gave no test, while group B gave a very strong one (approximately as much as the surrounding sea water). The cells were turgid and seemed normal in every respect. Hence, as far as we can judge from these experi-

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225.

[2] Rapkine and Wurmser have also reported the injection of dyes into the vacuole of *Spirogyra*, using the method of Chambers as modified by the Needhams (Rapkine, L., and Wurmser, R., *Proc. Roy. Soc. London, Series B*, 1927, cii, 128).

ments, the protoplasm can tolerate a considerable amount of sulfate on the vacuolar as well as on the outer surface.

It is also evident that there is no mechanism for rapidly disposing of sulfate after it has entered. If, therefore, the cells have in the past suffered mechanical or other injury which temporarily increased the permeability of sulfates, recovery would still leave a certain amount of these in the vacuole, without subsequent injury to the cell.

SUMMARY.

Potassium chloride and sulfate were injected into the vacuole of *Valonia*. The surviving cells tolerated the presence of these solutions. Sulfate, although ordinarily absent from the sap, is not rapidly eliminated when introduced. Hence the sulfate occasionally found in cells of normal appearance may have entered due to temporary injury followed by recovery.

INTERNAL VERSUS EXTERNAL TOXICITY IN VALONIA.

By A. G. JACQUES and W. J. V. OSTERHOUT.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, June 5, 1928.)

Our bioelectric measurements indicate that in *Nitella* and *Valonia* the protoplasm consists of layers,[1] of which the inner is more sensitive to the action of chloroform than the outer (when both are in contact with the same electrolytes at the same concentration).[2]

It is a matter of considerable interest to determine the relative sensitivity of these layers by applying a toxic substance to each one separately. The experiments here described were made for this purpose. The cells were divided into two groups. Those of group *A* were allowed to stand in sea water containing a small amount of the toxic agent so that only the outer layer (*X*) was directly exposed. In group *B* sap containing the toxic agent was injected into the cells.[3] Here only the inner layer (*Y*) was directly exposed. It seems probable that both *X* and *Y* are relatively impermeable to the toxic agent here employed ($MnCl_2$), at least as long as the protoplasm is not severely injured, and if this is the case we may expect death to occur more rapidly when the poison is applied to the more sensitive layer.[4]

The experiments were carried out in Bermuda on cells of *Valonia macrophysa* (containing from 0.3 to 1.2 cc. of sap) at about 20–25°C.

It was necessary to find a toxic agent which could be measured with a fair degree of accuracy even in very small amounts. Such organic reagents as chloroform or formaldehyde were ruled out because of analytical difficulties. After a number of trials in which Ni^{++}, Cd^{++}, Cu^{++}, Fe^{+++}, Cr^{++}, and Mn^{++} were investigated our choice fell on the last. From the analytical point of view Mn is excellent, since

[1] It is assumed that there is an inner layer *Y* and an outer layer *X* both of which are probably non-aqueous: between them there is an aqueous layer *W*.

[2] *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 673.

[3] *Cf.* Blinks, L. R., *J. Gen. Physiol.*, 1928–29, xii, 207.

[4] But if the protoplasm were freely permeable to the toxic agent it would make little difference whether it is injected into the cell or applied from the outside.

it can be determined to 0.01 mg. with good accuracy. It has, however, several valences (2, 3, 4, 6, and 7). In our experiments it was employed in the form of the bivalent salt, $MnCl_2$. This may be considered as the salt of a fairly strong base and a strong acid since it undergoes no appreciable hydrolysis. A possible objection is the uncertainty in regard to oxidation. In the sea water the chance of oxidation must be very small. On the other hand we know very little about the oxidation conditions within the vacuole of the cell. We do know, however, that the normal oxidation potentials of the electrodes

$$\left.\begin{matrix} Mn^{IV} \\ Mn^{II} \end{matrix}\right| Pt + 15\ N\ H_2SO_4 \quad \text{and} \quad \left.\begin{matrix} Mn^{III} \\ Mn^{II} \end{matrix}\right| Pt + 15\ N\ H_2SO_4$$

are strongly positive: according to Grube and Huberich[5] over 1.5 plus referred to the hydrogen electrode taken as zero. This indicates such a strong tendency of the higher valence compound to be reduced that it does not seem likely that the cell can oxidize $MnCl_2$.

It was hoped at the start that we might be able to determine the maximum concentration of Mn^{++} within the vacuole which the cell would tolerate indefinitely.[6] This was not realized. A considerable number of cells were found which fulfilled the conditions required, but on analysis they were found to contain so little Mn that the results were very inaccurate. In 17 out of 20 cases where the cells lived for a long time not more than a trace was found. For a similar reason we were unable to determine very accurately the maximum concentration of $MnCl_2$ in the sea water that the cell would tolerate indefinitely. The plan was therefore adopted of injecting the cells in group *A* with sap containing $MnCl_2$, and allowing them to stand in sea water until dead.[7] Analyses were then made of the cell contents. In this way data were obtained giving the length of life of cells containing 0.004 to 0.185 per cent of $MnCl_2$ in their sap. In group *B* the cells (stabbed with a capillary but not injected with $MnCl_2$) were simply placed in sea water containing $MnCl_2$ and allowed to remain until dead.

Technique.

In a previous paper[8] we have stated that occasionally cells which had been impaled on a capillary and then shaken from the capillary into sea water, healed

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[5] Grube, G., and Huberich, K., *Z. Elektrochem.*, 1923, xxix, 8.

[6] "Indefinitely" is here used in a limited sense. We know that cells will live for periods of months in sea water in the laboratory, without showing any change in the protoplasm except a slight fading of the color. In this work "indefinitely" simply indicates a period of a month or more during which there is no more change in the appearance than would be displayed by a cell not exposed to Mn.

[7] The sense in which this word is employed is explained on page 215.

[8] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, xi, 202.

up, becoming as turgid as unpunctured cells and continuing to live. This observation formed the basis of our present work. It was found that the proportion of punctured cells which could be made to heal could be very greatly increased by careful handling. Glass capillaries were used, and these were attached to a Luer hypodermic syringe of 2 cc. capacity. The capillaries were considerably finer than those used by us in making the measurements of potential difference. The glass at the tip was extremely thin, and the point was broken off at an angle to facilitate the piercing of the relatively tough cellulose wall. During injection the syringe was filled with sap containing a definite concentration of $MnCl_2$. The cell was then grasped firmly between the thumb and forefinger and quickly impaled on the capillary and at the same time a slight pressure was applied to the plunger of the syringe. As soon as the cell was in place on the capillary it was

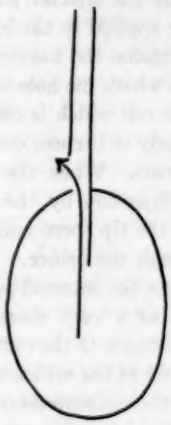

FIG. 1.

FIG. 2.

FIGS. 1 and 2. Methods of inserting a hollow steel needle into the cell.

relinquished by the fingers, and then a considerable pressure was applied to the piston. At this stage, those cells which showed any sign of leakage around the capillary were rejected. This was done because any severe leak indicated that an undue displacement of the natural sap by artificial sap plus $MnCl_2$ had probably occurred, and also because when a cell leaked on puncturing there was usually some scouring and tearing of the protoplasm at the site of the leak. This was probably due to the sudden rush of sap out through the minute annular space. Cells so damaged usually failed to heal. The passage of sap + $MnCl_2$ into the cell could be observed owing to the difference of refractive index of the solutions. It was thus possible to control in a very rough way the amount injected. It is obvious that at the moment of injection the pressure in the vacuole must have been above normal. Nevertheless in only a very few cases (excepting those cells which leaked at the puncture) was there any evidence of rupture of the cell wall.

Further it is of interest to note that those cells which showed any sign of such rupture were invariably large, containing 0.75 cc. or more. The actual transfer of the solution from the syringe to the cell took only a few seconds. While the pressure was still maintained the cell was brushed quickly from the capillary into a large volume of sea water.

This method of injection yielded the greatest number of cells which healed. Deflation of the cell was completely avoided. A second method which was tried and abandoned involved the use of a very fine hollow steel needle. This was ground off at the point at a steep angle. The cell was brought to the needle and forced up on it rather slowly. As Fig. 1 shows there was at the start a passage through which sap could escape as indicated by the arrow. This passage was closed as soon as the impalement was complete (Fig. 2). By applying a slight pressure to the piston the loss of sap was made up by the injected solution. The advantage was that no excess of pressure had to be applied to the interior of the cell, and that after practice it was possible to determine the amount of entering solution quite accurately by controlling the speed at which the hole was closed up. This plan, however, involved some deflation of the cell which is often fatal. If the cell wall is creased or folded the protoplasm is likely to become detached at this point, and this disturbance spreads until death occurs. When the cell is transferred from the capillary to the sea water after injection by the first method described, it is obvious that the moment it leaves the tip there must be a fall in the internal pressure by the escape of liquid through the orifice. Undoubtedly the time of outflow would be very short, both because the internal pressure would be high, and the orifice (which may be regarded as a very short capillary of relatively great diameter) would offer very small resistance to the outflow. Hence we should anticipate a great acceleration of the liquid at the orifice but we should not expect any great scouring of the protoplasm at the circumference for owing to the size of the opening the greatest acceleration would be opposite the center of the orifice, decreasing outward to the circumference. This is in marked contrast to the case previously discussed, where the flow was through a tiny annular orifice. Here owing to the smallness of the opening the greatest acceleration would be opposite the edges. It is probable that most of the material which escaped from the cell when the pressure fell was natural sap; for the injected sap plus $MnCl_2$ had a greater density and would tend to fall to the bottom of the cell away from the site of the puncture. This could actually be observed in certain preliminary experiments where the pink Co ion was injected. The ideal condition would have been to have released the pressure on the piston at the moment the cell left the capillary. This, however, could not be realized in practice and in consequence the exterior of the cell was subjected to a rain of the toxic solution at this time. However, by holding the cell just above the surface of a large beaker of sea water the length of this exposure was cut down and after injection the cell was removed from the beaker at once and washed in a stream of sea water. This served to rock and rotate the cell, and helped to bring about a thorough mixing of the sap and the slightly heavier toxic solution which was injected. The cell was then

placed in a 50 cc. crystallizing dish containing fresh sea water. This was kept covered to prevent evaporation. Each cell was preserved in a separate crystallizing dish to avoid the effect of exosmosis from other cells.

It was recognized that before healing was complete a certain amount of $MnCl_2$ might escape from the cell by diffusion through the orifice. In order to minimize any effect that this might have on the exterior of the cell, the sea water was changed a few hours after injection and then daily until the cell was healed.

To apply the toxic solution to the exterior the cells were placed in sea water containing varying concentrations of $MnCl_2$, 1 cell to a dish. Weighed amounts of $MnCl_2\ 2H_2O$ were added to sea water, but owing to the uncertain water content, the solutions so made up were analyzed for manganese.

It is plain that when the solution is injected into the cell we must consider not only the action of the toxic solution of the layer *Y* but also the effect of the puncture on the protoplasm. An attempt was made to eliminate the effect of stabbing by trying to determine the percentage of cells dying as the result of the stab alone but this was abandoned in favor of the plan of impaling all cells. All the cells of group *B* before being placed in the sea water containing $MnCl_2$ were injected with artificial sap containing no Mn. The possibility of the diffusion of $MnCl_2$ through the orifice into such cells must be considered. We might have permitted each cell to heal in sea water before placing it in the toxic sea water. But it was felt that this would give an advantage to these cells in comparison with those of group *A*, which were exposed to the Mn from the moment of injection.

Our experiments were carried out in two series, the following routine being observed. A few cells were injected with sap containing $MnCl_2$, washed, and placed in separate vessels of sea water, then the syringe was washed out with distilled water and finally with artificial sap. Then the same number of cells were injected with artificial sap, washed, and transferred to separate vessels of sea water containing $MnCl_2$. Finally a smaller but proportional number of cells were injected with the sap containing no $MnCl_2$, and these were placed, after washing, in sea water to serve as controls. This process was repeated, until the required number of cells for the series had been obtained. By proceeding in this way we made certain that the cells in each group were injected under exactly comparable conditions.[9]

It was not possible to control, except very roughly, the concentration of $MnCl_2$ applied to the interior of the cell. A small measure of control could be exercised by varying the concentration of the $MnCl_2$ in the injected sap. In practice two solutions were used, containing respectively about 0.05 N and 0.25 N $MnCl_2$. These concentrations represented a compromise. When a solution 0.01 N was injected into 25 cells 18 of them lived for more than 2 weeks without sign of injury. At the end of this period the experiment was terminated. On analysis only 6

[9] The needles were of course very fragile and when one broke during the stabbing the cell was rejected.

cells showed more than a trace of $MnCl_2$ present, while in two cases no Mn was detected. On the other hand, when a solution containing 0.1 N $MnCl_2$ was injected into a group of 9 cells, 8 of them failed to heal and within 48 hours the protoplasm had detached itself from the cellulose wall.

During the first 2 days after injection all the cells were examined carefully for signs of healing. In this respect the behavior of the control cells was of great interest. Many of these showed definite signs of recovery in 6 hours, and in nearly all cases healing was complete (as far as the eye could detect) within 24 to 48 hours. The visible signs of healing are the deposit of a black material in the puncture and the regaining of full turgidity.[10] Most of these cells continued to live indefinitely. A group of such healed cells compared with a group of unpunctured cells kept under similar conditions failed to show an appreciably higher mortality in 20 days. The effect of impalement is apparently shown at once, and if impaled cells heal they will survive almost as well as unpunctured cells. This conclusion is in line with our previous experiments[8] on potential difference in which cells were impaled and left on capillaries in sea water for periods up to a month, without suffering any apparent injury.

We believe that our procedure eliminated the effect of the impalement. All cells which persistently remained soft were rejected, and all cells which showed complete or even partial healing were kept (by partial healing we mean that a few cells showed the deposition of the black material in the puncture but died before they had completely recovered their turgidity).

It was observed that in the presence of Mn a series of changes took place with considerable regularity. Sometime after the sealing of the puncture, the protoplasm showed long thin areas of a lighter color. These widened, and became still lighter or even colorless. This process continued until the protoplasm was without color except for very small dark irregular patches. During these changes the cell retained its usual turgidity. At length it softened abruptly. At this stage the protoplasm either detached itself spontaneously from the cellulose wall or the detachment occurred when the cell was gently rolled between the fingers. These changes took place in both group *A* and group *B* when the concentration of the $MnCl_2$ was high. At lower concentrations the appearance of the lighter areas was either very much delayed or if they appeared early they spread comparatively slowly. In these cases the protoplasm usually detached itself spontaneously and the "colorless" and "soft" stages were absent. Variations of these main types of behavior were also observed. It was therefore necessary to select a stage in the protoplasmic changes to serve as a reference point in comparing the length of

[10] About 7 per cent failed to heal. These remained soft and within 24 to 72 hours showed a definite detachment of the protoplasm from the cellulose wall. In many of these cases the protoplasmic structure was greatly altered, and in others on gentle rolling between thumb and forefinger the protoplasmic layer broke up.

life of the cells. Only two points seemed suitable, namely: the first appearance of the light streaks, and the detachment of the protoplasm. The first of these was less desirable for it was not always possible to distinguish slight irreguarities present at the start from the incipient streakiness. The detachment of the protoplasm was therefore selected as our reference point. There was no detectable diffusion of Mn^{++} or $SO_4^{=}$ into the cell (and no diffusion of Mn^{++} from the cell) at any stage preceding the detachment of the protoplasm. A few hours after this had occurred, however, sufficient diffusion of these ions across the membrane had taken place to be readily detected. It would therefore seem as though this is the point at which the protoplasm becomes freely permeable. In the discussion which follows we shall designate cells which have reached this point as "dead."[11]

Analyses.

It was not, of course, possible to tell the exact moment when the protoplasm became freely permeable in cases where its detachment was spontaneous and hence in cells of group *A* it was necessary to take into account $MnCl_2$ which might have passed out into the sea water by diffusion. In making the analyses, therefore, the sea water was tested for Mn with the delicate reagent potassium periodate. When Mn was detected, care was taken to extract all the sap from the cell. This was done by piercing it with a fairly fine capillary pipette and by applying considerable suction. The external pressure caused the cell to collapse completely, and drove not only all the sap but also the detached protoplasm into the pipette. The sap was blown from the pipette into a small glass-stoppered bottle and weighed. A certain amount of inaccuracy was therefore introduced into the analysis since the weight would be too great by the weight of the protopasm. On the other hand since some of the Mn had passed out into the sea water the weight of sap from this cause was too little. We cannot accurately estimate these errors which tend to compensate each other, nevertheless it does not seem that they could have affected the final results very seriously. After the sap had been weighed it was added to the sea water in which the cell had stood and the solution was evaporated with 0.5 cc. of concentrated HNO_3, to oxidize the protoplasm, and 0.5 cc. of concentrated H_2SO_4, to change all the chlorides to sulfates. As an extra precaution, because of the large amount of chloride present, a second evaporation with H_2SO_4 was performed. The Mn was then oxidized according to the well known bismuthate method, and compared with standards containing known amounts of Mn.[12] In the group *B* analyses, where no appreciable amounts of Mn were detected in the sea water bathing the cells, the sea water was not included for the analysis, and since it was not necessary to empty the cell com-

[11] See also page 210.

[12] For details of the analysis see Standard methods for the examination of water and sewage, American Public Health Association, Boston, 5th edition, 51.

pletely, sap free from protoplasm was obtained for analysis. We have not been able to detect in these two sets of group *B* results any systematic variation comparable with the natural variation of the cells themselves.

DISCUSSION.

The effect of the treatment is shown in Fig. 3. For group *A* (Mn applied externally) the figures were obtained by averaging the length of life of all the cells (usually 18) at each concentration. For

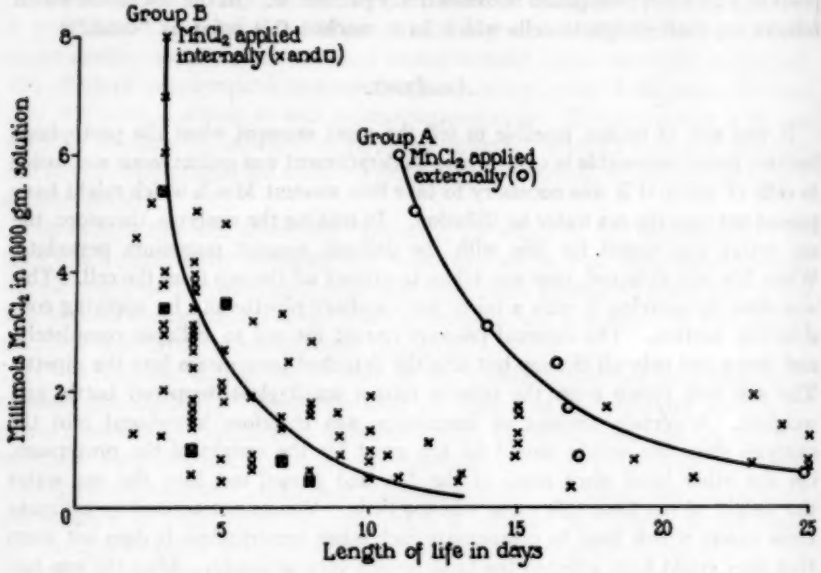

FIG. 3. Duration of life. The points for group *A* are obtained by averaging the length of life at each concentration. Those for group *B* are separate determinations of individual cells, the curve being drawn free-hand without attempting anything more than a rough fit.

group *B* (Mn applied internally) the length of life of each cell was plotted and a curve drawn free-hand without any attempt to do more than make a rough fit. The scattering of the points at low concentrations is to be expected since in general the application of dilute solutions of toxic substances gives very irregular results. (In general the controls lived more than a month after being stabbed.)

It is obvious that the cells of group *A* lived about twice as long as

those of group *B*. The difference might have been a little greater if the amount of Mn available for combination with the protoplasm had been the same in both cases; but where the Mn was applied externally at the same concentration as in the sap the external volume of solution was greater (10 to 15 cc. for each cell). Hence if the protoplasm combined with most of the Mn there would be more available in the latter case but it is not probable that more than a small fraction of the Mn was taken up by the protoplasm.[13]

Earlier in this paper we spoke of the possibility of the diffusion of $MnCl_2$ from the sea water into the cell before healing was complete. This might tend to reduce the average life of the cells by subjecting *Y* as well as *X* to the action of the toxic agent. This would tend to bring the curves closer together. Hence any error involved would not invalidate the conclusion that the injection of the $MnCl_2$ produces death more quickly than application to the exterior.

Hydrolysis[14] of $MnCl_2$ might affect the results by increasing the acidity but our tests indicate that this effect is negligible.

If we take the curves as drawn we see that, for example, when 0.0003 M $MnCl_2$ is applied internally the cells live about 12 days; to shorten the life of the cell to the same extent by an external application would require about 0.0045 M, a concentration more than 10 times as great.

The simplest interpretation might be to regard the inner layer, *Y*, as more sensitive than the outer, *X*, and to suppose that as soon as the inner layer is sufficiently altered to become permeable to $MnCl_2$ the latter diffuses through *Y* and attacks *X*. An attack from the outside would be slower because of the greater resistance of *X*.

An alternative assumption might be that *X* is permeable to the toxic agent and *Y* is not, and that the layer which is permeable is not

[13] It is of course possible that some Mn may combine with the cell wall in such a way as to be removed from the sphere of action but it does not seem probable that this is a factor of importance, especially as each cell was placed in a separate dish containing 10 to 15 cc. of solution.

[14] The hydrolysis of $MnCl_2$ appears to have been measured only once. Thus Kullgren (*Z. physik. Chem.*, 1913, lxxxv, 473; see Landolt-Börnstein, Physikalisch-chemische Tabellen, Berlin, 5th edition, 1923, ii, 1170) found by the sugar inversion method that a 0.25 M $MnCl_2$ solution at 100°C. was hydrolyzed only 0.0017 per cent.

injured by the $MnCl_2$ which diffuses through it so that when the toxic solution is injected *Y* will be attacked at once, but *X* will not be injured until after the alteration of *Y*.[15] When the toxic solution is applied to the outside of the cell *Y* will be subject to attack only after the toxic substance has diffused through *X*.

Even if there are differences of permeability it is probable that we also have differences of sensitivity as well to deal with. The assumption that *X* and *Y* are unlike is in harmony with our previous work on potential differences across the protoplasm of *Valonia*[16,17] and with experiments on the marine alga *Griffithsia*[18] and with the investigations of de Vries[19] in so far as they may be interpreted to mean that the inner and outer surfaces do not act alike. But the statement of Höber[20] that the inner and outer surfaces of the protoplasm may be unlike seems to apply rather to a difference between the inner and outer surfaces of *X*.

It is probably not worth while to attempt to compare our results with those of Chambers and his coworkers who have in some cases found much less toxicity when a substance is injected into *Amœba* than when applied to the outside,[21] since in these cases the toxic substance was not injected into a pre-existing vacuole as in the case of *Valonia* where the mechanism involved may be quite different.

[15] Throughout this part of the discussion the destruction of either layer is considered to be equivalent to death.

[16] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, xi, 193.

[17] Experimental evidence seems to indicate that *X* is actually permeable to certain ions, while *Y* may not be.

[18] Osterhout, W. J. V., *Science*, 1913, xxxviii, 408.

[19] de Vries, H., *Jahrb. wissensch. Bot.*, 1885, xvi, 465. See also Küster, E., *Ber. bot. Ges.*, 1909, xxvii, 589; *Arch. Entwcklngsmechn. Organ.*, 1910, xxx, pt. 1, 351 (Festschrift für Wilhelm Roux); *Z. Bot.*, 1910, ii, 689.

[20] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926, 732.

[21] A particularly striking case is that of picric acid, as described by Pollack and Howland (Pollack, H., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 145. Howland, R. B., and Pollack, H., *Proc. Soc. Exp. Biol., and Med.*, 1927, xxv, 221).

SUMMARY.

When $MnCl_2$ is injected into the cells of *Valonia macrophysa* they live only about half as long as when the same concentration is applied to the exterior of the cell. This is due to toxic action and not to the mechanical disturbance accompanying the injection (since all cells were stabbed in the same manner by the capillary).

A variety of explanations are suggested, all of which involve a difference between the inner and outer layers of the protoplasm.

STUDIES ON PERMEABILITY OF MEMBRANES.

VI. Mensuration of the Dried Collodion Membrane (Calculation of Dimensions and of Relations to Certain Biological Membranes).

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In a previous paper of this series a flat form of the dried collodion membrane and its method of preparation was described (1). As this form of membrane presented certain advantages in durability and convenience of manipulation over the previously used bag membranes, it was the type used in most of the subsequent investigations. Electric transfer experiments in which relatively strong electric currents were used (2, 3) and diffusion experiments continued over a period of several months (4) were both made possible because of the resistant and stable properties of these membranes. In experiments to be reported on in the near future the same type of membrane was used for conductivity measurements. In view of the many experiments made with these membranes it has seemed desirable to publish a brief account of measurements of the dimensions and physical properties of some of them. Such measurements are necessary when one wishes to compare diffusion rates, conductivity values, etc., with results obtained with other types of membranes. In the last paper of this series (4), dealing with the diffusion of non-electrolytes, the rates of diffusion of acetone, glycerol, urea and glucose were measured for a number of these membranes but as the result desired was a ratio of the rates at which different substances diffused it was not necessary to reduce the results with any one membrane to terms of unit area and thickness. Nevertheless some points of interest can be brought out by so doing as will be shown presently.

The membranes used in the present study were selected from those

previously used in the non-electrolyte diffusion experiments. The following measurements were made:

1. Area.—The membrane was removed from its bell-shaped glass supporting frame by cutting the attachment around the outside rim with a knife. With scissors the membrane was then trimmed in such a way as to remove as nearly as possible those bits of collodion whose only purpose was to glue the membrane to its frame. There remained a smooth, flat and approximately circular piece of collodion comprising at least most of the previously functioning surface. This was placed in water. For an estimation of the functioning area of the membrane it would have been sufficiently accurate to consider the membrane as a perfect circle and calculate the area from measurements of the diameter. However, as it was desired to use the exact area of the piece removed in calculating the membrane thickness, another method of obtaining the area was employed. A piece of good grade rather heavy white paper was selected and from it a square exactly 15 cm. on each side was cut. The weight of this piece of paper was determined. The membrane was then quickly blotted, placed on a piece of glass with the paper over it and the outline traced with a pencil. The tracing was repeated so that four outlines were made on the one piece of paper. These were now carefully cut out and weighed. The area of the piece of membrane was then calculated from the proportion.

$$\text{Membrane area: } 225 \text{ cm}^2. = \frac{\text{Weight of 4 tracings}}{4} : \text{Weight of } 225 \text{ cm}^2.$$

2. Weight of Wet Membrane.—By the weight of a wet membrane is meant the total weight of a membrane whose pore channels are still filled with water. Following the method of Hitchcock (5) each membrane was quickly blotted between filter papers, wiped dry of surface water, placed in a covered moisture dish and weighed. The average of several such weighings was taken as the correct weight. At first glance it would seem that such weighings must be extremely inaccurate because varying amounts of water might be removed from the pores by the filter paper. It is of interest, then, that the average deviation from the mean rate was never greater than 0.36 per cent and for all the membranes studied averaged 0.12 per cent.

3. Weight of Membrane Immersed in Water.—Several weighings were made of the membrane when it was suspended in water by a fine hair, the average figure being entered as the correct weight. The temperature of the water was recorded.

4. Weight of Dry Membrane.—After all other measurements had been completed the membrane was dried to constant weight in an oven at 60°C. This usually was reached in about 6 hours. With the type of collodion used it was not possible to dry at 100°C. as did Hitchcock. This temperature caused the collodion to become yellow and to lose weight steadily.

From the above measurements the following membrane dimensions were computed:

(a) Thickness: The weight of the wet membrane in air minus the weight when immersed in water equaled the displacement of water. The latter figure when corrected for temperature gave the volume of the membrane. The volume divided by the area gave the thickness. The average thickness of all the membranes studied was 0.0903 mm. This method of obtaining an accurate estimate of the thickness of a thin membrane is essentially the same as that used by Bjerrum and Manegold (6). These authors did not determine the weight of their membranes suspended in water but by using in their calculations a fixed value for the density of collodion reached the same result.

(b) Average cross-section area of pores: On the assumption that the weight of the wet membrane is merely equal to the weight of the dry membrane plus the weight of the water within the channels the latter weight was found and from it by temperature correction (in this case of negligible importance) the volume of the pores was computed. The pore volume divided by the membrane thickness gave an estimate of the average pore area on cross-section. Whether the channels are sufficiently uniform in calibre to allow this figure to be regarded as the area at any cross-section, and more especially the pore area at the two surfaces, it is of course not possible to say.

(c) Part of membrane occupied by pores: The proportion of the membrane occupied by pores was determined by finding the ratio between the pore volume and the volume of the whole membrane. The same percentage result could also be obtained by finding the ratio between the pore area and the area of the whole membrane. For the membranes studied it was found that the pores occupied from 10.5 per cent to 15.4 per cent of the entire membrane (average 13.1 per cent). In studying a series of collodion membranes of varying permeability but of the usual type used for dialysis Hitchcock (5) found that the more permeable membranes of his series were composed of 7 to 8 times as much water as collodion while the less permeable contained half as much water as collodion. In this respect then the figures given here represent a continuation of Hitchcock's series, the dried type of membrane being much less permeable and containing a much smaller proportion of water.

(d) Density of the wet membrane: The density of the wet membrane was determined by dividing the weight of the wet membrane by

its volume (loss of weight in water corrected for temperature). This figure represents the density of the membrane as a whole and in the form that it has been used in our experiments. It is of course a component result depending on the density of dry collodion and of water and of the proportions of the two in the membrane. In our series it varied from 1.567 to 1.602 and averaged 1.583.

(e) Density of the dry membrane: This figure represents merely the density of the dried collodion from which the membranes were made. As the data already collected permitted its estimation for each membrane, it was determined in each case, the result being a check on the accuracy of the original measurements. The weight of the membrane immersed in water subtracted from the weight of the membrane after drying gave (after the usual temperature correction) the volume of the collodion framework of the membrane exclusive of the pore volume. The dry weight divided by this volume gave the density. In the present series the variations were from 1.669 to 1.675, the average density being 1.672. The average deviation from the mean was only 0.11 per cent. The figure is in satisfactory agreement with that of 1.653 given by Hitchcock (5) for the collodion used in his experiments and of 1.72 for that used by Bjerrum and Manegold (6).

DISCUSSION.

The various measurements enumerated in the foregoing paragraphs have been tabulated in Table I. Although we have given these measurements principally to place on record the dimensions of the membranes used in our various experiments, still a few items of interest may be brought out in connection with them.

In the last paper of this series in publishing the results of diffusion experiments the amounts of acetone, urea, glycerol and glucose passing the membranes were compared. The results for each membrane were not expressed in terms of unit thickness and area as only the ratio between the rates holding for two different substances was being studied. In explaining the large differences in the diffusion rates between the substances of larger and smaller molecular size, it was supposed that the rate of diffusion of each substance was proportional to a certain "available pore area." The available pore area differed from the total pore area by the amount of pore area distributed among

holes too small to permit the passage of that substance. Glucose diffused more slowly than glycerol and glycerol many times more

TABLE I.

Membrane	Area	Thickness	Density wet	Density dry	Total area of pores at any cross-section	Part of membrane occupied by pores
	cm.²	*mm.*			*cm.²*	*per cent*
F-1	29.491	0.1056	1.572	1.673	4.401	14.9
F-2	28.930	0.1185	1.584	1.670	3.720	12.9
F-3	31.112	0.0894	1.581	1.670	4.113	13.2
F-5	32.847	0.0950	1.594	1.674	3.880	11.8
F-7	30.043	0.1036	1.589	1.672	3.704	12.3
F-8	27.871	0.0681	1.602	1.674	2.934	10.5
F-9	29.779	0.0886	1.567	1.669	4.486	15.1
F-10	30.275	0.0816	1.593	1.673	3.607	11.9
F-11	26.462	0.1044	1.591	1.671	3.120	11.8
C-6	30.232	0.0766	1.570	1.675	4.655	15.4
C-7	27.514	0.0615	1.571	1.669	4.027	14.6

TABLE II.

Membrane No.	Diffusion of glycerol			Diffusion of acetone		
	Rate for entire membrane	Rate for unit portion of membrane	Average deviation from mean unit rate	Rate for entire membrane	Rate for unit portion of membrane	Average deviation from mean unit rate
			per cent			*per cent*
F-1	1.71	0.410		123.0	29.48	
F-2	1.50	0.477		83.2	26.47	
F-3	1.56	0.339	43.6	125.3	27.20	13.3
F-5	0.78	0.191		76.8	18.79	
C-6	7.04	1.157		167.4	27.52	
C-7	2.84	0.433		243.5	37.13	

In the first column for each substance is given the diffusion rate for each of the membranes as previously determined. In the second column this rate has been standardized by multiplying by the membrane thickness (expressed in units of 0.1 mm.) and dividing by the cross-section pore area. It will be seen that the standardized rates are considerably more uniform in the case of acetone than with glycerol.

slowly than acetone because a much larger percentage of the total pore area was available for the substances of smaller molecular size.

In other words it can be inferred from this theory that as the molecular size of the diffusing substance decreases the value of the available pore area will approach that of the total pore area. It would then seem that in studying the diffusion rate of substances of small molecular size one might hope to find some relation to the total pore area whereas with substances of larger molecular size no such relation should exist. The experiments with acetone and glycerol seemed best suited for testing this assumption. Eight membranes were used in these experiments; six were measured by the method described in this paper. In making this test the values for the diffusion rates of acetone and glycerol given in the last paper (4) (Table IV) were reduced to terms of unit thickness (0.1 mm.) and unit pore area (1.0 sq. cm.) by using the dimensions given in Table I of this paper. The results are listed in Table II. It will be seen that according to expectation the standardized rates for acetone are much more uniform than those for glycerol. With glycerol the average deviation from the mean result is 43.6 per cent while with acetone it is only 13.3 per cent. Better agreement than this could scarcely be expected for it must be remembered that according to the theory some part of the total pore area must be taken up by pores too small to permit the passage even of acetone.

It has already been mentioned that one purpose in recording these measurements is to make possible comparisons with other types of membranes and especially with biological membranes. Such comparisons are desirable and even necessary to determine within what limits the dried collodion membrane may be taken as a model for very much more complicated cell membranes. Unfortunately peculiar obstacles stand in the way of him who would determine the dimensions of cell membranes. These membranes are extremely thin and delicate and any kind of direct measurements are impossible. Within recent years several investigators, notably Fricke (7) and McClendon (8), have described a method of approaching the problem indirectly. Both of these investigators have estimated the thickness of the red blood cell membrane from measurements of the electric capacity made with high frequency currents. At best the values given must be regarded as approximations only, for even if the factor of experimental error be eliminated they depend upon assumed values

for the dielectric constant of the membranes. Nevertheless, the method is extremely ingenious and is the best approach to a difficult problem that has been made so far. McClendon has calculated the cell membrane thickness as 3×10^{-8} cm. if he assumes a dielectric constant of 3 and as 3×10^{-7} cm. if he takes the dielectric constant as 10. Fricke, assuming a dielectric constant of 3, but with capacity measurements somewhat different from those of McClendon, calculated the thickness as 3.3×10^{-7} cm. In other words the membrane thickness is almost within the range of molecular dimensions. Recognizing the approximate nature of these figures it is interesting to use them for making some rough comparisons between the dried collodion membrane and the membrane of the red blood cell.

In previous papers there have been enumerated some of the potential effects observable when the dried collodion membrane represents the interphase in concentration chains and in chemical chains. It will be recalled that these potential differences do not depend on the thickness of the interphase but merely on the nature and concentration of the adjacent solutions and on certain properties of the membrane itself. On the other hand the force acting on any charged particle within the membrane depends on the intensity of the electric field at this point and the field intensity does depend on thickness. The electric field intensity (ϵ) is given by the formula

$$\epsilon = \frac{E}{d}$$

where E is the potential difference between the two borders of the membrane and d the membrane thickness. It is thus evident that, for a given potential difference, the thinner the membrane the greater the intensity of the electric field and consequently the greater the force acting upon a charged ion within its borders. If we take the thickness of the average dried collodion membrane of the type used in these experiments as 1×10^{-2} cm. and that of the red blood cell membrane as 3×10^{-7} cm. it is evident that equal potential effects will in the case of the cell membrane give rise to an electric field with an intensity 30,000 times greater than in the case of the collodion membrane. In other words very small electromotive effects may have a large significance with biological membranes.

In previously reported experiments in which the transfer number of chlorine in several chloride solutions in different concentrations was determined by means of direct electric transfer experiments it was shown that the electric current itself was capable of altering appreciably the transfer number that existed at the moment of first application of the current. Even with currents of 2 to 4 milliamperes, which were used in most of the experiments, the results of electric transfer experiments were in certain ranges of concentration quite measurably different from transfer numbers estimated for the same membrane from concentration chains. Inasmuch as the rate at which a given electrolyte can diffuse across a membrane depends upon the transfer numbers of its ions (being a maximum when they are each equal to 0.5) it follows that an applied electromotive force can materially change the rate of diffusion of a substance dissociated into ions. For example with one membrane in a medium concentration range with KCl an applied electromotive force of 1 volt was necessary to establish a current of 4 milliamperes across the membrane. Supposing that this membrane was roughly 0.1 mm. thick, this is equivalent to an electric field intensity of 100 volts per cm. To establish the same electric field intensity across the membrane of the red blood cell (thickness taken as 3×10^{-7} cm.) would require a potential difference of only 0.03 millivolt. Potential differences of this magnitude and greater must frequently arise in the animal organism. In fact E. J. Warburg (9) has estimated that a difference in pH of from 7.2 to 7.4 between cells and plasma may give rise to a potential difference of 2 millivolts. It is possible though of course only a surmise that such electromotive effects arising as a result of nerve impulses or because of changes in the concentration of the tissue fluids may be of paramount importance in controlling rates of excretion and secretion by certain cells and be intimately associated with such complex phenomena as the chloride shift.

From the standpoint of the diffusion of non-electrolytes it is interesting to see in how far the dried collodion membrane might serve as a model of a cell membrane if its dimensions were similar. At first glance the recorded rates for the diffusion of glucose with these collodion membranes seem extremely slow and this slowness seems at variance with the rapidity with which the amount of glucose on the

two sides of the red blood cell membrane may become equalized. It will be recalled that with the collodion membranes the fastest rate recorded was only 1.57×10^{-5} millimols per hour for the whole membrane with a diffusion pressure of 2.4 atmospheres. This amounts to 1.56×10^{-6} mg. per minute per sq. cm. of membrane surface at the same pressure. The thickness of this membrane was 7.7×10^{-3} cm. Now it has been estimated that in 1 cc. of blood there are 5,000 sq. cm. of membrane surface (8). If we again take the thickness as 3×10^{-7} cm. and suppose that some physiological event has raised the plasma glucose 72 mg. per 100 cc. above the cell glucose (producing a diffusion pressure of 0.1 atmosphere at 37 C.) we can calculate that if the substance of the red blood cell membrane were dried collodion glucose would diffuse across the membrane at a rate of 8.5 mg. per minute per cc. of blood as long as the pressure was maintained. That is, the entire 72 mg. per 100 cc. originally present as an excess in the plasma could have diffused across the cell membranes in about 5 seconds. Of course the pressure is not maintained for as diffusion continues the pressure falls. But at least we can say that within several minutes the difference in concentration between cells and plasma would no longer be detectable and this is quite in accord with what little is known concerning the rapidity with which glucose may be exchanged between cells and plasma across the normal red blood cell membrane.

SUMMARY.

The flat type of dried collodion membrane used by Michaelis and his associates in numerous investigations has been subjected to mensuration in order that the dimensions of these membranes may be placed on record. The membranes had a functioning area of about 30 cm., were approximately 0.1 mm. in thickness and were composed on the average of 87 per cent by volume of collodion and 13 per cent by volume of pores.

In reviewing some of the previously reported results of diffusion experiments with non-electrolytes in the light of the calculated values for the total pore area for the same membranes additional evidence was presented to show that a smaller molecule (acetone) probably utilizes a much larger percentage of the total pore area for its diffusion than is available for a larger molecule (glycerol).

By using the figures of Fricke and McClendon for the thickness of the membrane of the red blood cell some comparisons were drawn between the dried collodion membrane as a model for certain biological membranes and the red blood cell membrane. In these comparisons emphasis was placed on the exaggerated importance of small electromotive forces and very slight permeabilities when these were associated with membranes of such extreme thinness as the red blood cell membrane.

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9. Warburg, E. J., *Biochem. J.*, 1922, xvi, 329.

THE PREARGININE IN EDESTIN AND ITS RESISTANCE TO HYDROLYSIS.

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I.

INTRODUCTION.

In a previous paper[1] it was shown that the amount of arginine occurring as such ($pK' = 8.1$) in proteins is a small portion of the arginine found on hydrolysis. The remainder is produced by the hydrolysis of a weak basic group ($pK' = 4.6$) to which the name "prearginine" is given.[2]

II.

Edestin.

We now turn to edestin (a protein from hemp) which gives a high yield in arginine on hydrolysis. The available data on edestin in alkaline solution are inconsistent and we obtained new titration data in this range. Since our new data are no marked improvement over the old we will not publish them but present our analysis of the data assembled by Cohn.[3] Curve *A* in Fig. 1 shows the experimental values. The dotted portion of Curve *A* (in alkaline solution) indicates that the protein is modified in that range. The true curve in alkaline solution should agree with Curve B_3.

Curve B_1 corresponds to the amino acids found on hydrolysis. In

[1] Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 629.

[2] The name "prearginine" is given to that portion of the protein molecule having a basic group ionizing about pH 4.6 and yielding arginine on hydrolysis. It may be composed of one, or of more than one, amino acid. Such a weak basic group should consist of a primary amino group attached to a conjugated unsaturated system (perhaps cyclic) as in aniline or cytosine.

[3] Cohn, E., *Physiol. Rev.*, 1925, v, 349.

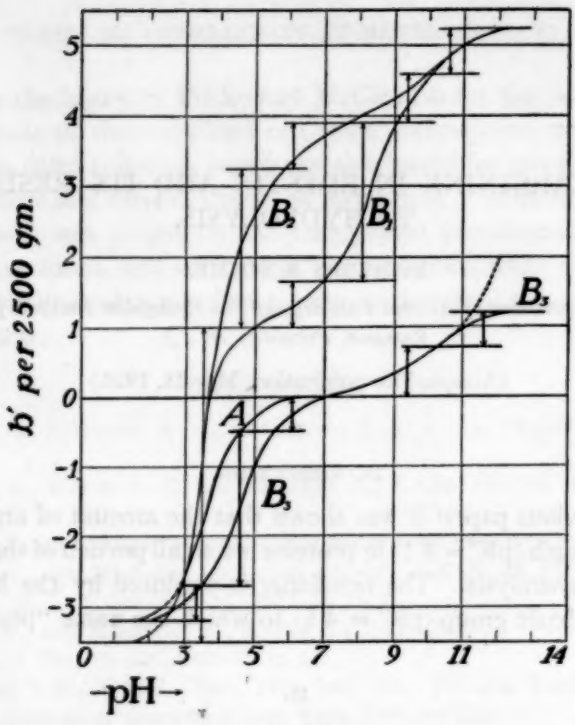

FIG. 1. *A*. Experimental titration curve of edestin. B_1, B_2, B_3, curves calculated to correspond with various amounts of the ionizable groups.

TABLE I.

Analysis of Edestin Data Demonstrating the Relation between Arginine and Prearginine.

Groups	Sources	Indices in edestin†	Equivalents per 2,500 gm.		
			On hydrolysis	Titration data	Difference
Acidic	Dicarboxylic acids*	$pG_1' = 3.0$†	4.5*	0.5	−4.0
	Tyrosine	$pG_2' = 9.4$	0.7	0.7	0
Basic	**Prearginine**	$pG_3' = 3.6$†	0	2.2	+2.2
	Histidine	$pG_3' = 5.1$†	0.6	0.5	−0.1
	Arginine	$pG_4' = 8.1$	2.2	0	−2.2
	Lysine	$pG_5' = 10.6$	0.6	0.5	−0.1

* The value for the free carboxyl groups is the total dicarboxylic acids minus the amount bound as amides (7.8 − 3.3 = 4.5 equivalents).

† The indices of carboxyl groups, of prearginine and of histidine in *gelatin* are about 3.5, 4.6 and 6.1 respectively.

B_2 there is no arginine but an equal amount (2.2 equivalents) of prearginine. B_2 represents the same groups but in the amounts given in Table I (under "titration data"). This last curve (B_3) is probably correct on the alkaline side but must be shifted to the left on the acid side in order to agree with the experimental data (Curve *A*). This indicates that the COOH index is a little lower than 3.5 (say 3.0) and the prearginine and histidine indices are lower than their usual values (about 3.6 and 5.1).

The results of the titration curve analysis are given in the next to the last column of Table I. The preceding column gives the equivalents of the amino acids found on hydrolysis. The values for histidine and lysine (0.6 and 0.6) are taken from Van Slyke[4] rather than from Vickery and Leavenworth[5] since the latter values (0.3 and 0.4) are lower than those we obtain from titration data (0.5 and 0.5).

It will be observed that there is *no free arginine group in edestin* and that all the 2.2 equivalents found on hydrolysis *exist in this protein as prearginine.* 4.0 equivalents of the "free" carboxyl groups do not ionize (perhaps bound as anhydride). All the other groups *i.e.*, tyrosine, histidine and lysine exist in edestin in approximately the amounts found on hydrolysis.

III.

Hydrolyzed Edestin.

We made up four samples of edestin and hydrolyzed them with pepsin in acid solution (data given in Tables II to V). The hydrolyses were stopped at different points, neutralized with an amount of alkali exactly equal to the acid and titrated electrometrically.

Edestin contains 18.6 per cent nitrogen, or 33.3 mols per 2,500 gm. 9.0 equivalents of this is non-α-nitrogen in the basic groups, leaving 24.3 equivalents of α-nitrogen. The initial acid-combining capacity (due to the ionizable non-α-nitrogen) is 3.2 equivalents. Hence the degree of hydrolysis given by the equation

$$\text{Per cent hydrolysis} = \frac{100\ (A - 3.2)}{24.3}$$

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1911, x, 15.

[5] Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1928, lxxvi, 707.

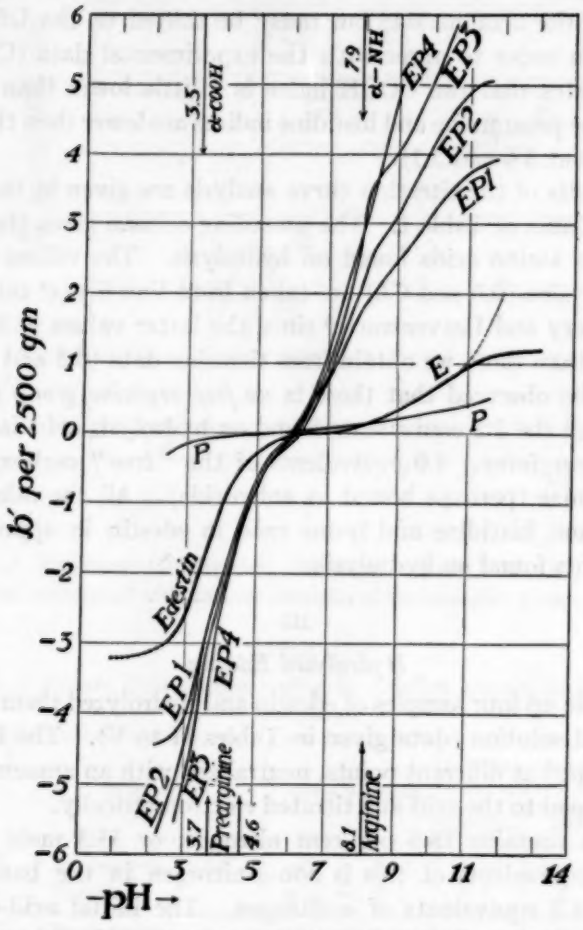

FIG. 2. Titration curves of edestin (*E*), of pepsin (*P*) and of solutions of edestin hydrolyzed to different degrees with pepsin (EP_1, EP_2, EP_3, and EP_4).

where A is the acid-combining capacity (in equivalents per 2,500 gm.) of a hydrolyzed solution. On this basis the four samples of edestin were hydrolyzed 5 per cent, 14 per cent, 18 per cent and about 30 per cent respectively. These were titrated and the data are given in Tables II to V.

None of these solutions showed any change of prearginine into arginine in their titration data (given in Fig. 2). Such a change would be manifested by a drop in the *EP* curves at 3.7 (prearginine) and a rise at 8.1 (arginine), as compared with the Curve *E* of edestin. Comparison curves[6] were drawn and in the first three samples corroborated this conclusion (that prearginine is not converted into arginine in these solutions). However the comparison curve of the fourth sample showed that it had hydrolyzed so far that the buffer effect of the α carboxyl groups (3.3) and the α amino groups (7.9) would make it impossible to observe this change if it occurred.

Thus we know that prearginine is not destroyed by hydrolysis up to 18 per cent with pepsin. We also know that complete hydrolysis destroys it, since arginine can be isolated quantitatively from the resulting solution.[5]

Hunter[7] has shown that the rate of hydrolysis of edestin with trypsin indicates that arginine exists in two forms. We would suppose that the more resistant form is prearginine except that the data (Table II) indicate, in this particular protein, that there is no free arginine group (all existing as prearginine). Felix[8] showed that part of the "arginine" from hydrolyzed proteins was not precipitated in the usual manner. This fraction may be prearginine. Edlbacher[9] separated fractions high in arginine from oxidized proteins. Part of these arginine-rich fractions resisted further hydrolysis. The significance is doubtful.

[6] Simms, H. S., and Levene, P. A., *J. Biol. Chem.*, 1926, lxx, 319. The curves in Fig. 2 would be more accurate if the curve for the corresponding amount of pepsin were subtracted from the observed curve, but this would not affect the conclusions. Titration data of pepsin are given in Table VI.

[7] Hunter, A., *Trans. Roy. Soc. Canada*, 1925, xix, 1.

[8] Felix, K., *Z. physiol. Chem.*, 1922, cxx, 94.

[9] Edlbacher, S., *Z. physiol. Chem.*, 1924, cxxxiv, 129.

IV.

Growth-Promoting Activity.

Dr. Lillian E. Baker kindly tested the growth-promoting activity[10] of the first three of the above samples of hydrolyzed edestin, on sarcomatous fibroblasts. They showed activity which was essentially the same in all three samples. The fourth sample was not tested.

V.

EXPERIMENTAL.

The hydrolyses of edestin were carried out as follows: 3,750 gm. edestin in about 125 cc. water, plus the designated volume of M HCl, plus the designated weight (0.2–0.5 gm.) of Armour's 1:10,000 pepsin was allowed to stand the designated lengths of time at 37°C.

After hydrolysis the HCl was neutralized with the same volume of M NaOH and the solutions were made up to 150 cc. (2.5 per cent edestin solution). The solutions were heated to 90°C. and filtered from the slight precipitate which formed on heating. These solutions were titrated by adding requisite amounts of dilute acid or base to 5 cc. samples, making up to 10 cc. and determining the pH. The final solutions were 1.25 per cent with respect to edestin, corresponding to a total volume of 300 cc.

[10] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503. Baker, L. E., and Carrel, A., 1928, xlvii, 353, 371.

TABLE II.

Titration Data of Sample EP_1 of Edestin Hydrolyzed with Pepsin.

(6 cc. M HCl; 0.2 gm. pepsin; 3.5 hours at 37°C., giving 5 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
2.174	−6.0	0.050	−4.448	6.3	0	0.020	0
2.580	−5.0	0.045	−4.393	6.323	0.25	0.022	0.250
3.161	−4.0	0.040	−3.842	6.663	0.50	0.023	0.500
3.294	−3.5	0.038	−3.384	6.792	0.75	0.024	0.750
3.585	−3.0	0.035	−2.941	7.316	1.0	0.025	1.000
3.819	−2.5	0.033	−2.465	7.653	1.5	0.027	1.500
4.103	−2.0	0.030	−1.982	7.936	1.75	0.029	1.750
4.381	−1.5	0.027	−1.491	8.296	2.0	0.030	2.000
4.755	−1.0	0.025	−0.996	9.061	2.5	0.033	2.497
5.007	−0.75	0.024	−0.748	9.729	3.0	0.035	2.984
5.215	−0.50	0.023	−0.500	10.941	4.0	0.040	3.739
5.472	−0.25	0.022	−0.250	11.561	5.0	0.045	3.905
6.046	−0.125	0.021	−0.125	11.827	6.0	0.050	3.965

TABLE III.

Titration Data of Sample EP_2 of Edestin Hydrolyzed with Pepsin.

(6 cc. M HCl; 0.2 gm. pepsin; 23 hours at 37°C., giving 14 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
2.252	−7.0	0.055	−5.698	6.355	0.25	0.022	0.250
2.558	−6.0	0.050	−5.356	6.626	0.50	0.023	0.500
2.792	−5.0	0.045	−4.628	6.989	0.75	0.024	0.750
3.301	−4.0	0.040	−3.885	7.208	1.0	0.025	1.000
3.457	−3.0	0.035	−2.920	7.457	1.5	0.027	1.500
3.892	−2.5	0.033	−2.471	7.905	2.0	0.030	2.000
3.981	−2.0	0.030	−1.976	8.161	2.5	0.033	2.500
4.399	−1.5	0.027	−1.491	8.817	3.0	0.035	2.998
4.771	−1.0	0.025	−0.996	9.805	4.0	0.040	3.982
5.068	−0.50	0.023	−0.492	10.866	5.0	0.045	4.779
5.479	−0.25	0.022	−0.250	11.475	6.0	0.050	5.095
6.16	0	0.020	0	11.814	7.0	0.055	5.015

TABLE IV.

Titration Data of Sample EP_3 of Edestin Hydrolyzed with Pepsin.

(4.5 cc. M HCl; 0.5 gm. pepsin; 23 hours at 37°C., giving 18 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
2.724	−6.0	0.052	−5.552	6.636	0.5	0.025	0.500
3.123	−5.0	0.047	−4.826	7.074	1.0	0.027	1.000
3.395	−4.0	0.042	−3.907	7.374	1.5	0.030	1.500
3.685	−3.0	0.037	−2.953	7.634	2.0	0.032	2.000
3.945	−2.5	0.035	−2.474	7.851	2.5	0.035	2.500
4.220	−2.0	0.032	−1.986	8.005	3.0	0.037	3.000
4.443	−1.5	0.030	−1.492	8.174	3.5	0.040	3.499
4.810	−1.0	0.027	−0.996	8.963	4.0	0.042	3.998
5.26	−0.5	0.025	−0.499	9.469	4.5	0.045	4.491
6.2	0	0.022	0	9.946	5.0	0.047	4.973
				10.611	6.0	0.052	5.876

TABLE V.

Titration Data of Sample EP_4 of Edestin Hydrolyzed with Pepsin.

(6 cc. M HCl; 0.5 gm. pepsin; 94 hours at 37°C., giving roughly 30 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
3.583	−4.0	0.042	−3.948	6.575	0.50	0.025	0.500
3.869	−3.0	0.037	−2.969	6.946	1.0	0.027	1.000
4.244	−2.0	0.032	−1.987	7.480	2.0	0.032	2.000
4.846	−1.0	0.027	−0.997	8.098	3.0	0.037	3.000
5.213	−0.50	0.025	−0.499	8.544	4.0	0.042	3.999
5.614	−0.25	0.025	−0.250	9.012	5.0	0.047	4.997
5.771	0	0.022	0				

TABLE VI.

Titration of 0.067 Per Cent Pepsin Solution.

(0.2 gm. per 150 cc. mother solution or 300 cc. final solution; treated like the other solutions but containing no edestin.)

pH	$\frac{b-a}{c}$	b'	pH	$\frac{b-a}{c}$	b'
2.491	−1.0	−0.355	7.223	0.08	0.080
2.539	−0.8	−0.222	8.201	0.16	0.160
2.680	−0.6	−0.182	8.580	0.2	0.200
2.927	−0.4	−0.127	10.357	0.4	0.341
3.389	−0.2	−0.118	10.920	0.6	0.386
3.580	−0.1	−0.047	11.167	0.8	0.422
5.408	0	0	11.349	1.0	0.425
			11.631	1.6	0.500

VI.

SUMMARY.

The titration data of edestin show that all the arginine found on hydrolysis exists in this protein as "prearginine."[1,2]

The extra ionizable groups of histidine, lysine and tyrosine are free in the quantities found on hydrolysis. Part of the extra carboxyl groups of aspartic and glutamic acids are bound as amides, and 50 per cent are bound in some other manner (perhaps anhydride) leaving only about 6 per cent of these groups free to ionize in edestin.

The prearginine in edestin is not converted into arginine on hydrolysis with pepsin up to 18 per cent (of the total hydrolysis). In more highly hydrolyzed solutions it is not possible to detect such a conversion, due to high buffering. Complete hydrolysis however converts prearginine into arginine which can be isolated.

Hydrolyzed edestin promotes the growth of sarcomatous fibroblasts about equally well whether 5, 14 or 18 per cent hydrolyzed.

CHEMICAL ANTAGONISM OF IONS.

I. Effect of Na-Mg and K-Mg Mixtures on the Activity of Oxalic Dion.

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I.

INTRODUCTION.

It has long been known that various inorganic ions produce characteristic and specific effects on living organisms; and that in so far as the effects of two or more ions are opposite in character, they neutralize (or "antagonize") each other when present in the same solution. In this brief introductory paper it is inadvisable to review the literature on the subject, but it is sufficient to mention that interest particularly concerns four cations, namely; sodium, potassium, calcium, and magnesium.

In general the effect of sodium and potassium is to cause relaxation on one hand and greater permeability on the other. Calcium and magnesium cause contraction and impermeability. But there are also differences between the effects of sodium and potassium and between those of calcium and magnesium.

It has been found that colloids behave differently with different cations. In general calcium and magnesium precipitate sols while sodium and potassium stabilize sols. Antagonism between the effect of univalent and divalent cations on emulsions has been studied by van der Meulen and Rieman[1] and on arsenious sulfide sols by Weiser;[2]

[1] van der Meulen, P. A., and Rieman, W., *J. Am. Chem. Soc.*, 1924, xlvi, 876. Rieman, W., and van der Meulen, P. A., *J. Am. Chem. Soc.*, 1925, xlvii, 2507.

[2] Weiser, H. B., Colloid symposium monograph, New York, 1926, iv, 354.

while Höber[3] and others have studied antagonism in other colloidal systems. While these are interesting, they deal with unknown factors and have failed to produce a satisfactory explanation of the biological phenomena.

The data below show that a very definite antagonism between sodium and magnesium is found in true (non-colloidal) solutions of oxalate. These agree with simple mathematical formulas. It is hoped they may throw some light on the physiological and on the colloidal antagonisms.

II.

Theory.

In another publication[4] we showed that the *monoions* of monovalent and polyvalent weak acids in the presence of Na^+ or Mg^{++} ions behave normally in that they obey the limiting Debye-Hückel equation in dilute solution.

Diions and *triions* of weak acids in the presence of Na^+ were found to obey a modified equation in which we introduced a correction for the distance between the like charges. However the presence of Mg^{++} causes marked decrease in the activity of the diion (or triion) which cannot be explained by the Debye-Hückel theory.

TABLE I.

Inactivation of Oxalate Diion by Mg^{++}, as Shown by Conductivity Data.

Data on 0.001 molar $MgCl_2$ and Na_2Ox solutions and on a mixture of equal parts of these solutions. The ionic strength was equal to $\mu = 0.003$ in all three solutions.

Solution	Conductivity × 10^3	Mean
$MgCl_2$	21.1	16.5
Na_2Ox	11.9	
1–1 mixture	14.4	14.4
Difference.....		2.1 = 13 per cent decrease

[3] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 5th edition, 1924, pp. 587–692.

[4] Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.

The deviation of diions with Mg^{++} is small (0.8) for sebacic and azelaic acids. It is greater (1.4 and 8.3 respectively) for succinic and malonic acids, and very great (17) for oxalic acid. The effect on citric acid diion (9) and triion (29) are also large. Oxalic acid was chosen to work with owing to its high deviation and the fact that the difference between its titration indices ($pG_2 - pG_1$) is large enough to greatly simplify the calculations (which are complex enough at best).

Inactivation of dianions with Mg^{++} can be shown by conductivity data, as demonstrated in Table I. The conductivity of a mixture of $MgCl_2$ and $NaSO_4$ solutions is 13 per cent less than the mean of the two solutions.

The antagonism experiments consisted in determing the pH of solutions of oxalic acid containing about 1.5 equivalent of NaOH to which various amounts of $MgCl_2$ and NaCl (or KCl) were added. By calculating the pK′ value and comparing it with that of a similar solution with the same ionic strength but containing NaCl alone (*i.e.*, no

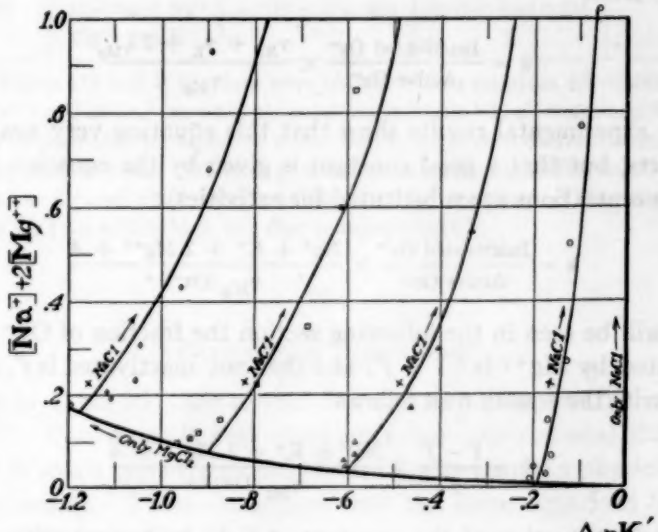

FIG. 1. Effect of $MgCl_2$ and of $MgCl_2$-NaCl mixtures on pK_2' of oxalic acid (corrected for the "normal" effect produced by the same ionic strength of NaCl). $\Delta_T pK_2'$ values are equal to $pK_2' - pK_T'$, see Fig. 2.

$MgCl_2$), it was possible to determine the effect of Mg^{++} ion on the activity of the oxalate diion.

We found that with a given amount of $MgCl_2$ the deviation was larger in the absence of NaCl or KCl. Addition of NaCl or KCl caused a decrease in this deviation, and the more NaCl or KCl added the greater the decrease until the value approached the "normal" value (with NaCl or KCl alone). Fig. 1 clearly demonstrates the effect of only $MgCl_2$ or of $MgCl_2$ + NaCl.

This constitutes a definite *chemical antagonism between ions* in pure, non-colloidal solution.

III.

Formulation of the Antagonism.

If we assume that the Mg^{++} ion combines with the oxalate diion ($Ox^{=}$) to inactivate it and that the Na^{+} ion (or K^{+} ion) when present interferes with this combination we would expect the following equation to hold:

$$k = \frac{\text{Inactivated } Ox^{=}}{\text{Active } Ox^{=}} \times \frac{\gamma_{Na} + \gamma_{K} + 2\,\gamma_{Mg}}{\gamma_{Mg}} \qquad (1)$$

The experimental results show that this equation very nearly fits the facts, but that a good constant is given by the equation (where ion concentrations are substituted for activities):

$$k = \frac{\text{Inactivated } Ox^{=}}{\text{Active } Ox^{=}} \times \frac{Na^{+} + K^{+} + 2\,Mg^{++} + A}{\gamma_{Mg}\,(Ox^{=})^{1.5}} \qquad (2)$$

As will be seen in the following section the fraction of $Ox^{=}$ ion inactivated by Mg^{++} is $(1 - f')$ and that not inactivated is f', and we may write the equation as follows:

$$k = \frac{1 - f'}{f'} \times \frac{Na^{+} + K^{+} + 2\,Mg^{++} + A}{\gamma_{Mg}\,(Ox^{=})^{1.5}} \qquad (3)$$

The introduction of the constant A (which changes with oxalate concentration) and of the concentration of oxalate diion raised to the 1.5 power, renders the equation semi-empirical rather than theoretical. The equation is nevertheless useful in proving that the observed an-

tagonism follows definite laws even down to zero concentration of Na^+ and K^+.

The agreement of the data with equation (3) is summarized in Table II in which the empirical values of A are given and also the calculated values of k. The agreement of the k values may be seen in Tables III, V, VII, and VIII.

TABLE II.

Cation Antagonism.

Calculation of $k = \left(\frac{1-f'}{f'}\right)\frac{Na^+ + K^+ + 2\ Mg^{++} + A}{\gamma_{Mg}\ (Ox=)^{1.5}}$.

Table No.	Cations present	Approximate concentration of oxalate	Empirical constant A	$k \cdot 10^{-4}$
III	Mg^{++} and Na^+	M/100	0.290	15
V	"	M/200	0.060	16
VII	Mg^{++} and K^+	M/100	0.12	9
"	"	M/200	0.02	10
VIII*	Mg^{++} and Na^+	M/100 and M/200	(0.031) and 0.023	22*
" *	Mg^{++} and K^+	"	"	18*

* In Tables III and V the data were obtained from solutions all containing at least 0.0075 mol of Na^+ ion while the solutions in Table VII all contained K^+ ions. In order to show that the equation would hold down to zero concentration of Na^+ or K^+ the data in Table VIII were obtained from a series including solutions free from Na^+ or K^+ and which were found to obey the equation. (The values of k in Table VIII are a little high, but that is unimportant.)

IV.

Physiological Significance.

Data in Table VIII are carried down to zero concentration of Na^+ and K^+. This point is important since our experimental data are mostly in much higher concentrations of salts than the physiological concentrations. These data show that the same equations hold in the physiological range.

We have demonstrated[4] two types of deviations of the activity of weak electrolytes caused by the presence of Mg^{++} ions, namely the effect on weak cations and the effect on weak polyanions. We have

also shown[5] that Mg^{++} ions produce deviations in the activity of proteins (presumably due to both effects). Our present data show that the effect on polyanions is antagonized by Na^+ and K^+ ions and there should be a corresponding antagonism in protein activity.

Such an effect on protein activity may be involved in the physiological antagonisms of ions. Unfortunately we were unable to use Ca^{++} ions in our experiments due to insolubility of calcium oxalate. The effect should be similar to that with Mg^{++}. The following paper will show that a similar antagonism exists between anions (SO_4^- and Cl^-).

We furthermore studied solutions containing NaCl and KCl (but no $MgCl_2$). Although Na and K give slightly different activities, the difference is too small to demonstrate an antagonism in a mixed solution (see Table VI).

V.

Experimental and Mathematical.

A mother solution of oxalic acid was made up containing the indicated equivalents of NaOH. To 5 cc. samples of this, varying quantities of 0.25μ and 2.5μ solutions of NaCl (or KCl) and $MgCl_2$ were added and the solutions diluted to 10 cc. (0.01026 molar oxalate). The concentrations of Mg^{++} and Na^+ are given in Tables III to VIII. The concentrations of Na^+ due to NaOH added are included in the indicated values for Na^+.

b' was calculated from the formula:[6]

$$b' = \frac{b - a}{c} + \frac{h}{c} \tag{5}$$

where h is given by the concentration index (ph) which differs from the activity index (pH) by an amount varying with the ionic strength.[4]

pK_2' is obtained by the equation:

$$pK_2' = pH - \log \frac{\alpha_2}{1 - \alpha_2} \tag{6}$$

where $\alpha_2 = b' - 1$.

[5] Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 613.

[6] Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

pK_T' is the index of a similar solution of the same ionic strength and containing NaCl but free from $MgCl_2$. The pK_T' values were observed from a plot of NaCl data given in Table IV. See Fig. 2.

$\Delta_T pK_2' = pK_2' - pK_T'$ is the deviation produced by Mg^{++} ions.

The activity coefficient f' is the antilogarithm of $\Delta_T pK_2'$. It represents the relative amount of active diion. The actual amount

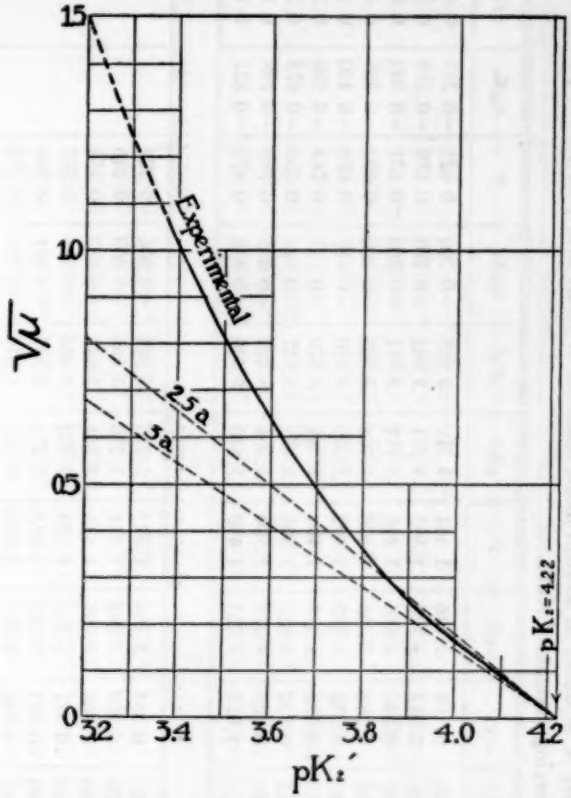

FIG. 2. Effect of NaCl on pK_2' of oxalic acid. This effect is assumed to be "normal" and the abnormalities produced by other salts are measured in terms of their deviation from this curve. Values of pK_2' read from this curve are called "pK_T'."

We have plotted the values of pK_2' against the square root of the ionic strength ($\sqrt{\mu}$). Curves are also given for the limiting Debye-Hückel equation ($pK_2 - pK_2' = 3a\sqrt{\mu}$) and for the modified equation[4] ($pK_2 - pK_2' = 2.5a\sqrt{\mu}$).

TABLE III.

Antagonistic Effect of Na^+ and Mg^{++} on the Activity of Oxalic Diions.

Calculation of k (where $A = 0.290$) in 0.01026 molar solutions of oxalic acid plus 1.462 equivalents of NaOH with fixed amounts of $MgCl_2$ and varying amounts of NaCl.

$Mg^{++} \times \frac{1}{1000}$	$(\alpha c)^{1.5} \times \frac{1}{1000}$	Na^+	$\sqrt{\mu}$	pH	b'	pK_2'	pK_T'	$\Delta_T pK_2'$	f'	$\Delta_0 pK_2$	f_0	$\gamma_{Mg} \times \frac{1}{1000}$	$k \times 10^{-4}$
4.17	0.364	0.015	0.180	3.746	1.481	3.779	3.985	−0.206	0.622	−0.235	0.582	3.04	18
"	"	0.028	0.212	3.730	1.482	3.761	3.942	−0.180	0.659	−0.278	0.527	3.24	14
"	"	0.040	0.240	3.686	1.485	3.711	3.911	−0.200	0.631	−0.309	0.491	3.24	16
"	"	0.065	0.288	3.681	1.486	3.705	3.865	−0.160	0.692	−0.355	0.441	3.47	(12)
"	"	0.140	0.398	3.587	1.492	3.600	3.770	−0.170	0.676	−0.450	0.355	3.58	15
"	"	0.265	0.532	3.534	1.496	3.541	3.670	−0.127	0.743	−0.550	0.282	3.80	14
"	"	0.515	0.730	3.433	1.505	3.425	3.547	−0.122	0.755	−0.673	0.212	3.90	18
"	"	0.765	0.885	3.379	1.509	3.364	3.462	−0.098	0.798	−0.758	0.175	3.99	18
"	"	1.015	1.017	3.323	1.490	3.340	3.398	−0.058	0.875	−0.822	0.151	4.07	13
Average..													16
16.7	0.386	0.015	0.264	3.284	1.522	3.246	3.886	−0.604	0.229			16.7	17
"	"	0.028	0.288	3.292	1.521	3.256	3.865	−0.609	0.246			"	16
"	"	0.040	0.308	3.297	1.525	3.261	3.846	−0.585	0.254			"	16
"	"	0.065	0.357	3.263	1.520	3.220	3.804	−0.584	0.255			"	17
"	"	0.140	0.443	3.313	1.523	3.276	3.737	−0.461	0.346			"	13
"	"	0.265	0.566	3.282	1.526	3.242	3.648	−0.406	0.392			"	14
"	"	0.515	0.755	3.248	1.526	3.203	3.534	−0.331	0.467			"	15
Average..													15

41.7	0.43	0.015	0.382	2.972	1.587	2.819	3.784	−0.965	0.108			41.7	18
"	"	0.028	0.395	2.983	1.584	2.835	3.773	−0.938	0.115			"	17
"	"	0.040	0.414	2.986	1.583	2.840	3.758	−0.918	0.121			"	16
"	"	0.065	0.443	3.003	1.579	2.864	3.737	−0.872	0.133			"	16
"	"	0.140	0.520	3.016	1.575	2.884	3.680	−0.796	0.160			"	15
"	"	0.265	0.629	3.035	1.569	2.914	3.609	−0.685	0.207			"	13
"	"	0.515	0.803	3.016	1.572	2.889	3.506	−0.617	0.242			"	16
"	"	0.765	0.946	2.983	1.577	2.848	3.432	−0.584	0.261			"	18
"	"	1.015	1.070	2.947	1.584	2.799	3.373	−0.574	0.267			"	21
Average													15
83.3	0.51	0.015	0.520	2.764	1.654	2.487	3.680	−1.193	0.064			83.3	16
"	"	0.028	0.532	2.805	1.637	2.561	3.670	−1.109	0.078			"	13
"	"	0.040	0.543	2.797	1.640	2.547	3.663	−1.116	0.077			"	14
"	"	0.065	0.566	2.822	1.628	2.595	3.648	−1.053	0.088			"	13
"	"	0.140	0.628	2.817	1.640	2.567	3.610	−1.043	0.091			"	13
"	"	0.265	0.721	2.830	1.634	2.592	3.552	−0.960	0.110			"	14
"	"	0.515	0.878	2.808	1.638	2.566	3.466	−0.900	0.126			"	16
"	"	0.765	1.010	2.776	1.645	2.517	3.401	−0.894	0.128			"	19
Average													15
Average of all values													15

TABLE IV.

Effect of NaCl Alone on pK_2' of Oxalic Acid.

NaCl added	Na^+	$\sqrt{\mu}$	pH	b'	pK_2'
A. (0.0126 molar oxalic acid plus 1.462 equivalents of NaOH plus varying amounts of NaCl)					
0	0.015	0.141	3.989	1.473	4.036
0.013	0.028	0.182	3.930	1.475	3.973
0.025	0.040	0.212	3.901	1.476	3.942
0.050	0.065	0.265	3.841	1.479	3.877
0.125	0.140	0.381	3.754	1.482	3.785
0.250	0.265	0.520	3.653	1.488	3.673
0.500	0.515	0.722	3.543	1.495	3.551
0.750	0.765	0.878	3.460	1.502	3.459
1.000	1.015	1.010	3.409	1.505	3.401
1.125	1.140	1.070	3.385	1.507	3.373
B. (0.00513 molar oxalic acid plus 1.462 equivalents of NaOH)					
0	0.0075	0.100	4.049	1.481	4.082
0.0025	0.0100	0.112	4.035	1.482	4.066
0.0050	0.0125	0.123	4.028	1.483	4.057
0.0075	0.0150	0.132	4.006	1.483	4.035
0.0100	0.0175	0.142	3.989	1.484	4.016
0.025	0.0325	0.187	3.945	1.487	3.967
0.050	0.057	0.245	3.883	1.492	3.896
0.075	0.082	0.292	3.861	1.494	3.870
0.100	0.107	0.332	3.832	1.496	3.835

TABLE V.

Antagonism of Na^+ and Mg^{++} in Dilute Solutions.

Calculation of k (where $A = 0.060$) in 0.00513 molar oxalic acid plus 1.462 equivalent of NaOH with fixed amounts of $MgCl_2$ and varying amounts of NaCl.

$\frac{Mg^{++}}{1000}\times$	$\frac{(ac)^{1-3}}{1000}\times$	Na^+	$\sqrt{\mu}$	pH	b'	pK_1'	pK_2'	$\Delta_2 pK_1'$	f'	$\Delta_0 pK_1$	f_0	$\frac{\gamma_{Mg}}{1000}\times$	$k \times 10^{-4}$
1.67	0.118	0.0075	0.123	3.852	1.492	3.865	4.047	−0.182	0.66	−0.17	0.68	1.12	(28)
"	"	0.0100	0.132	3.906	1.489	3.925	4.037	−0.117	0.76	−0.18	0.66	1.29	16
"	"	0.0125	0.142	3.901	1.489	3.920	4.022	−0.102	0.79	−0.20	0.63	1.36	11
"	"	0.0150	0.149	3.895	1.490	3.912	4.011	−0.099	0.80	−0.21	0.62	1.39	12
"	"	0.0175	0.158	3.883	1.491	3.898	4.003	−0.105	0.78	−0.22	0.60	1.36	14
"	"	0.0325	0.200	3.824	1.496	3.831	3.949	−0.118	0.76	−0.27	0.53	1.36	14
"	"	0.057	0.255	3.808	1.498	3.811	3.884	−0.073	0.84	−0.34	0.45	1.50	13
"	"	0.082	0.300	3.790	1.506	3.790	3.853	−0.063	0.86	−0.37	0.43	1.53	13
"	"	0.107	0.339	3.752	1.503	3.747	3.819	−0.073	0.85	−0.40	0.40	1.53	17
Average													14
3.33	0.133	0.0075	0.142	3.801	1.496	3.808	4.022	−0.214	0.61	−0.20	0.63	2.73	13
"	"	0.0100	0.149	3.796	1.497	3.801	4.011	−0.210	0.62	−0.21	0.62	2.76	13
"	"	0.0125	0.158	3.790	1.499	3.791	4.003	−0.212	0.61	−0.22	0.60	2.78	14
"	"	0.0150	0.166	3.771	1.499	3.772	3.993	−0.221	0.60	−0.23	0.59	2.76	15
"	"	0.0325	0.212	3.754	1.502	3.751	3.937	−0.186	0.65	−0.28	0.53	2.88	14
"	"	0.057	0.265	3.734	1.504	3.729	3.859	−0.156	0.70	−0.33	0.47	3.00	14
"	"	0.082	0.308	3.717	1.506	3.707	3.847	−0.140	0.72	−0.37	0.43	3.05	14
Average													14

TABLE V—*Continued.*

$Mg^{++} \times \frac{}{1000}$	$(ac)^{1.5} \times \frac{}{1000}$	Na^{+}	$\sqrt{\mu}$	pH	b'	pK_1'	pK_T'	$\Delta_T pK_2'$	f'	$\Delta_0 pK_2$	f_0	$\gamma Mg \times \frac{}{1000}$	$k \times 10^{-4}$
8.33	0.140	0.0075	0.187	3.485	1.535	3.425	3.967	−0.542	0.29	−0.23	0.59	7.33	20
"	"	0.0100	0.194	3.511	1.531	3.457	3.958	−0.501	0.31	−0.26	0.55	7.43	19
"	"	0.0125	0.200	3.500	1.533	3.443	3.949	−0.506	0.31	−0.27	0.54	7.45	19
"	"	0.0150	0.206	3.494	1.534	3.435	3.943	−0.508	0.31	−0.28	0.53	7.45	19
"	"	0.0175	0.212	3.494	1.534	3.435	3.935	−0.500	0.32	−0.28	0.52	7.50	19
"	"	0.0325	0.245	3.519	1.531	3.465	3.900	−0.435	0.37	−0.32	0.48	7.62	19
"	"	0.057	0.292	3.524	1.531	3.470	3.858	−0.388	0.41	−0.36	0.44	7.71	17
"	"	0.082	0.332	3.512	1.533	3.455	3.823	−0.368	0.43	−0.40	0.40	7.78	19
"	"	0.107	0.367	3.531	1.531	3.477	3.794	−0.317	0.48	−0.43	0.37	7.88	19
Average													19
Total average													16

TABLE VI.

Effect of Mixtures of Na^+ and K^+ in Dilute Solutions.

(0.00513 molar oxalic acid plus 1.462 equivalents of NaOH with fixed amounts of KCl and varying amounts of NaCl).

K^+	Na^+	$\sqrt{\mu}$	pH	b'	pK_1'	pK_T'	$\Delta_T pK_1'$
0.0050	0.0075	0.123	4.006	1.483	4.035	4.047	−0.012
"	0.0100	0.132	3.966	1.485	3.991	4.037	−0.046
"	0.0125	0.142	3.978	1.485	4.001	4.022	−0.021
"	0.0150	0.149	3.996	1.484	4.023	4.011	+0.022
"	0.0175	0.158	3.962	1.486	3.986	4.003	−0.017
"	0.0325	0.200	3.934	1.488	3.954	3.949	+0.005
"	0.057	0.255	3.890	1.491	3.905	3.884	+0.021
"	0.082	0.300	3.854	1.494	3.864	3.853	+0.011
"	0.107	0.339	3.817	1.497	3.822	3.819	+0.003
0.0100	0.0075	0.142	3.994	1.484	4.021	4.022	−0.001
"	0.0100	0.149	3.983	1.485	4.008	4.011	−0.003
"	0.0125	0.158	3.974	1.485	3.999	4.003	−0.004
"	0.0150	0.166	3.971	1.486	3.995	3.993	+0.002
"	0.0325	0.212	3.922	1.489	3.941	3.937	−0.004
"	0.057	0.265	3.876	1.492	3.829	3.885	−0.056
"	0.082	0.308	3.851	1.495	3.859	3.847	+0.012
0.025	0.0075	0.187	3.932	1.488	3.952	3.967	−0.015
"	0.0100	0.194	3.925	1.489	3.944	3.958	−0.014
"	0.0125	0.200	3.918	1.489	3.937	3.949	−0.012
"	0.0150	0.206	3.910	1.490	3.927	3.943	−0.016
"	0.0175	0.212	3.915	1.489	3.934	3.935	−0.001
"	0.0325	0.245	3.879	1.492	3.892	3.900	−0.008
"	0.057	0.292	3.864	1.494	3.874	3.858	+0.016
"	0.082	0.332	3.820	1.497	3.825	3.823	+0.002
"	0.107	0.367	3.803	1.499	3.804	3.794	+0.010

TABLE VII.

Antagonism of K^+ and Mg^{++} in Solutions Free from Na^+.

Calculation of k in 0.00513 molar* and 0.01026 molar† oxalic acid plus 1.440 equivalent of KOH with fixed amounts of $MgCl_2$ and varying amounts of KCl.

$Mg^{++} \times \frac{1}{1000}$	$(ac)^{1-k} \times \frac{1}{1000}$	K^+	$\sqrt{\mu}$	pH	b'	pK_1'	pK_2'	$\Delta_2 pK_1'$	f'	$\Delta_0 pK_1$	f_0	$\gamma_{Mg} \times \frac{1}{1000}$	$k \times 10^{-4}$
1.67	0.12	0.0075	0.123	3.832	1.472*	3.881	4.048	−0.167	0.680	−0.17	0.68	1.14	11
"	"	0.0100	0.132	3.854	1.470*	3.906	4.035	−0.129	0.743	−0.18	0.66	1.26	8
"	"	0.0125	0.142	3.813	1.473*	3.860	4.022	−0.162	0.688	−0.20	0.63	1.19	11
"	0.32	0.0275	0.180	3.795	1.458†	3.868	3.973	−0.105	0.785	−0.25	0.56	1.11	11
"	"	0.040	0.212	3.774	1.459†	3.845	3.936	−0.091	0.811	−0.28	0.53	1.20	10
"	"	0.065	0.265	3.736	1.461†	3.803	3.883	−0.080	0.831	−0.34	0.46	1.29	9
"	"	0.140	0.381	3.654	1.466†	3.713	3.783	−0.070	0.851	−0.44	0.36	1.43	10
"	"	0.265	0.520	3.588	1.470†	3.640	3.679	−0.039	0.914	−0.54	0.29	1.44	8
Average.....													10
4.17	0.12	0.0075	0.149	3.668	1.487*	3.690	4.015	−0.325	0.473	−0.20	0.63	3.38	10
"	"	0.0100	0.158	3.703	1.484*	3.730	4.003	−0.273	0.533	−0.22	0.60	3.50	8
"	"	0.0125	0.166	3.661	1.488*	3.681	3.991	−0.310	0.490	−0.23	0.59	3.45	10
"	0.33	0.0275	0.212	3.715	1.462†	3.781	3.935	−0.154	0.701	−0.28	0.53	3.42	6
"	"	0.040	0.240	3.675	1.464†	3.737	3.906	−0.169	0.678	−0.31	0.49	3.42	7
"	"	0.065	0.287	3.651	1.466†	3.710	3.863	−0.153	0.703	−0.36	0.44	3.56	7
"	"	0.090	0.328	3.602	1.469†	3.656	3.827	−0.171	0.674	−0.39	0.41	3.51	9
"	"	0.140	0.397	3.590	1.470†	3.642	3.770	−0.128	0.745	−0.45	0.35	3.75	7
"	"	0.265	0.532	3.526	1.475†	3.569	3.668	−0.099	0.796	−0.55	0.28	3.89	8
"	"	0.515	0.730	3.464	1.480†	3.499	3.547	−0.048	0.895	−0.67	0.21	4.08	6
Average.....													8

8.33	0.14	0.0075	0.187	3.456	1.518*	3.425	3.965	−0.540	0.288	−0.25	0.56	7.37	11
"	"	0.0100	0.194	3.470	1.516*	3.443	3.956	−0.513	0.301	−0.26	0.55	7.39	10
"	"	0.0125	0.200	3.456	1.518*	3.425	3.948	−0.532	0.294	−0.27	0.54	7.42	10
"	0.36	0.0275	0.240	3.475	1.478†	3.513	3.906	−0.393	0.404	−0.31	0.49	6.97	10
"	"	0.040	0.265	3.477	1.478†	3.515	3.885	−0.370	0.427	−0.33	0.47	7.06	9
"	"	0.065	0.308	3.494	1.478†	3.534	3.846	−0.312	0.488	−0.37	0.43	7.30	10
"	"	0.090	0.346	3.467	1.480†	3.501	3.812	−0.311	0.489	−0.41	0.39	7.39	11
"	"	0.140	0.413	3.446	1.482†	3.477	3.760	−0.283	0.521	−0.46	0.35	7.53	9
"	"	0.265	0.543	3.411	1.483†	3.437	3.654	−0.217	0.607	−0.57	0.27	7.86	9
"	"	0.515	0.738	3.357	1.491†	3.372	3.542	−0.170	0.676	−0.68	0.21	8.00	11
Average													10
Total average 0.00513 molar* solutions													10
Total average 0.01026 molar† solutions													9

* 0.00513 molar oxalate, $A = 0.020$.
† 0.01026 molar oxalate, $A = 0.12$.

TABLE VIII.

Antagonism of Na^+ and Mg^{++} and of K^+ and Mg^{++} Including Solutions with No Na^+ or K^+.

Calculation of k in 0.0064 molar* solutions of oxalic acid plus 1.151 equivalents of MgO plus varying amounts of NaCl and KCl.

Mg^{++}	Na^+	$(\alpha c)^{1+\delta} \times \frac{1}{1000}$	$\sqrt{\mu}$	pH	b'	pK_1'	pK_T'	$\Delta_T pK_1'$	f'	$\Delta_{sp}K_2$	f_0	$\gamma_{Mg} \times \frac{1}{1000}$	$k \times 10^{-4}$
0.0037	0	0.177	0.183	2.971	1.246	3.457	3.956	−0.499	0.317	−0.26	0.55	2.5	
"	0	0.070	0.129	3.191	1.263	3.638	4.042	−0.404	0.394	−0.18	0.66	3.0	22
"	0.00125	"	0.134	3.192	1.263	3.639	4.031	−0.392	0.405	−0.19	0.65	3.0	22
"	0.0025	"	0.138	3.192	1.263	3.639	4.027	−0.388	0.409	−0.19	0.64	3.1	22
"	0.0050	"	0.147	3.198	1.262	3.647	4.015	−0.368	0.429	−0.20	0.62	3.1	22
"	0.0075	"	0.156	3.206	1.260	3.659	4.008	−0.349	0.448	−0.21	0.61	3.1	22
"	0.0125	"	0.171	3.206	1.261	3.657	3.984	−0.327	0.471	−0.24	0.58	3.2	21
"	0.025	"	0.208	3.208	1.263	3.655	3.938	−0.283	0.521	−0.28	0.52	3.3	22
"	0.050	"	0.258	3.218	1.262	3.667	3.889	−0.220	0.602	−0.33	0.47	3.4	22
"	0.075	"	0.303	3.220	1.263	3.667	3.848	−0.181	0.659	−0.37	0.42	3.5	22
Average													22
	K^+												
0.0037	0	0.177	0.183	2.971	1.246	3.457	3.956	−0.499	0.317	−0.26	0.55	2.5	18
"	0	0.070	0.129	3.191	1.263	3.638	4.042	−0.404	0.394	−0.18	0.66	3.0	18
"	0.00125	"	0.134	3.191	1.263	3.638	4.031	−0.393	0.404	−0.19	0.65	3.0	18
"	0.0025	"	0.138	3.194	1.262	3.643	4.027	−0.384	0.413	−0.19	0.64	3.1	18
"	0.0050	"	0.147	3.194	1.263	3.641	4.015	−0.374	0.423	−0.20	0.62	3.1	19
"	0.0075	"	0.156	3.201	1.261	3.652	4.008	−0.346	0.451	−0.21	0.61	3.1	18
"	0.0125	"	0.171	3.211	1.260	3.665	3.984	−0.319	0.480	−0.24	0.58	3.2	18
"	0.025	"	0.208	3.218	1.260	3.672	3.938	−0.266	0.542	−0.28	0.52	3.3	18
"	0.050	"	0.258	3.233	1.258	3.692	3.887	−0.195	0.638	−0.33	0.47	3.4	18
"	0.075	"	0.303	3.243	1.257	3.704	3.848	−0.144	0.718	−0.37	0.42	3.5	16
Average													18

* The first solution of both series is 0.0128 molar ($A = 0.031$) instead of 0.0064 molar ($A = 0.023$).

is $f'f\ \alpha_2 c$ and the actual amount of diion inactivated by Mg^{++} is $(1-f')f\alpha_2 c$, hence

$$\frac{\text{Inactivated diion}}{\text{Active diion}} = \frac{(1-f')f\ \alpha_2\ c}{f'\ f\ \alpha_2\ c} = \frac{1-f'}{f'} \tag{7}$$

In order to correct the concentration of Mg^{++} ion for the quantity removed in inactivation we must calculate

$$\Delta_0 pK_2 = pK_T' - pK_2$$

where $pK_2 = 4.220$ is the true index in infinite dilution.

The antilogarithm of $\Delta_0 pK_2$ is equal to f or the activity coefficient in the presence of the same ionic strength of NaCl. We then get the activity of Mg^{++} ion from the equation:

$$\gamma_{Mg} = Mg^{++} - (1-f')f\ \alpha_2\ c \tag{8}$$

The value of $(Ox^{=})^{1.5}$ is equal to $(\alpha_2 c)^{1.5}$ in the case of oxalic acid where the titration indices are isolated.

The values of k are calculated by equation (3) (see Tables II to VIII). In this equation we use concentration values of Na^+, K^+, and Mg^{++} in the numerator rather than the activity values indicated in equation (1), the latter being unnecessary.

VI.

SUMMARY.

Magnesium ions decrease the activity of divalent organic anions much more than the normal decrease produced by sodium ions. The effect is very large with short chain acids, particularly oxalic acid.

The addition of sodium or potassium ions produces a marked decrease in the effect of magnesium diions on the activity of oxalate diions.

Quantitative data on 0.005 molar solutions of oxalic diion over a wide range of concentrations of $MgCl_2$ and of NaCl (or KCl) show that the following equation is obeyed:

$$k = \frac{\text{Inactivated } Ox^{=}}{\text{Active } Ox^{=}} \times \frac{Na^+ + K^+ + 2\ Mg^{++} + A}{\gamma_{Mg}\ (Ox^{=})^{1.5}}$$

The ionic solution of both series is 0.0125 molar ($A = 0.031$) instead of 0.0064 molar ($A = 0.023$).

where A is an empirical value dependent on the concentration of oxalate diion ($Ox^{=}$).

This equation has been shown to hold down to zero ionic strength of Na^{+} and K^{+}, and hence to be valid in the physiological range.

These observations are of biological interest since the activity of proteins should (like oxalic acid) show a similar antagonism.

CHEMICAL ANTAGONISM OF IONS.

II. Antagonism between Anions and Also between Cations and Anions in Their Effect on Oxalate Activity.

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(Accepted for publication, May 20, 1928.)

I.

INTRODUCTION.

In the previous paper[1] a chemical antagonism between cations was demonstrated. $MgCl_2$ produces an anomalous depression in the activity of oxalate diions while NaCl or KCl produces a normal depression. The presence of NaCl or KCl antagonizes the anomalous effect of $MgCl_2$ according to the semi-empirical equation:

$$k = \frac{1 - f'}{f'} \frac{2Mg^{++} + Na^{+} + K^{+} + A}{\gamma_{Mg} \cdot (Ox^{=})^{1.5}} \quad (1)$$

where f' is the fraction of the oxalate diions ($Ox^{=}$) inactivated by Mg^{++} and $1\text{-}f'$ is the fraction not inactivated.

In this paper we will show that a similar behavior is displayed by Cl^{-} ions in antagonizing the effect of $SO_4^{=}$ ions on oxalate ionization (an effect opposite in direction from that of Mg^{++}). We will also show the result of having Mg^{++} and $SO_4^{=}$ in the same solution.

II.

DISCUSSION.

Previous papers[2] showed that sulfates cause a rise in the values of pK_1' and pK_2' of malonic acid above the normal indices required by the limiting Debye-Hückel equation.

[1] Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241.

[2] Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.

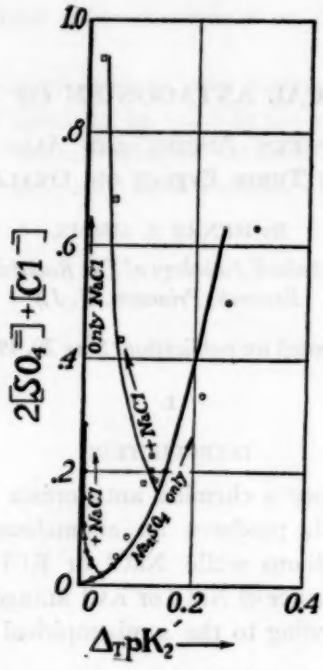

FIG. 1. Effect of Na_2SO_4 and of Na_2SO_4–NaCl mixtures on pK_2' of oxalic acid.

In the present study we find that the effect of $SO_4^=$ (alone) on pK_2' of oxalic acid increases with the concentration of $SO_4^=$ according to the equation:

$$k = (f' - 1)\frac{2}{\sqrt{SO_4^=}} \qquad (2)$$

This is shown in the right-hand curve of Fig. 1 which is drawn to fit equation (2) and agrees well with the data (circles). The value of k (=2.6) is calculated in Table II.

The presence of NaCl antagonizes the effect of Na_2SO_4 as shown in the curves of Fig. 1 for solutions containing fixed amounts of Na_2SO_4 and variable amounts of NaCl. These curves obey the equation:

$$k = (f' - 1)\frac{2SO_4^= + Cl^- + A}{(SO_4^=)^{1.5}} \qquad (3)$$

TABLE I.

Anion Antagonism.

Calculation of $k = (f' - 1)\frac{2SO_4 + Cl + A}{(SO_4)^{1.5}}$.

Table No.	Anions present*	Approximate oxalate concentration	$SO_4^=$ concentration	A	k
II	$SO_4^=$ only	M/100	0 to 0.333	0	2.6
III	$SO_4^=$ and Cl^-	M/100	0.0083	0.015	2.6
			0.0833	0.015	2.6
IV	$SO_4^=$ and Cl^-	M/200	0.0083	0.36	5.5
			0.0833	2.48	5.2

* Anions present in addition to the oxalate anions.

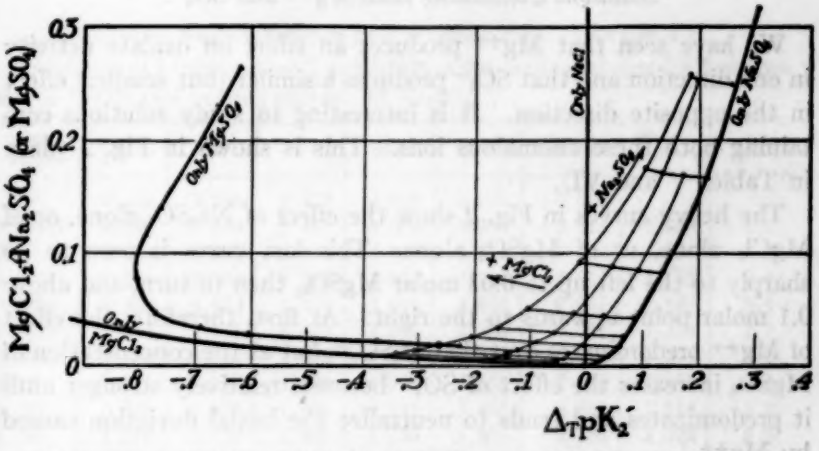

FIG. 2. Effect of Na_2SO_4, of $MgCl_2$, of $MgSO_4$, and of $Na_2SO_4 - MgCl_2$ mixtures on pK_2' of oxalic acid.

The $MgSO_4$ curve is not quantitatively comparable with the other curves due to the difference in amounts of Na^+ and Cl^- present.

The results are summarized in Table I. It will be noted that A is practically zero in all M/100 solutions. However in M/200 solution of oxalate A varies with the sulfate concentration, and is the only case we have observed in which A is not a constant with a

given oxalate concentration. The experimental data are given in Tables II to IV.

The similarity between the first terms of equations (2) or (3) and that of equation (1) is shown by the relation:

$$f' - 1 = \frac{1 - 1/f'}{1/f'} \tag{4}$$

The value of $f' = 10^{\Delta_I pK_2'}$ is less than unity in the data of equation (1), but greater than unity for the data of equations (2) and (3).

Thus we find that the effect of $SO_4^=$ on oxalate activity is antagonized by Cl^- in a manner similar to the antagonism by Na^+ or K^+ of the effect of Mg^{++}.

III.

Solutions Containing Both Mg^{++} and $SO_4^=$.

We have seen that Mg^{++} produces an effect on oxalate activity in one direction and that $SO_4^=$ produces a similar (but smaller) effect in the opposite direction. It is interesting to study solutions containing both these anomalous ions. This is shown in Fig. 2 (data in Tables V and VI).

The heavy curves in Fig. 2 show the effect of Na_2SO_4 alone, or of $MgCl_2$ alone, or of $MgSO_4$ alone. This last curve is seen to go sharply to the left up to 0.03 molar $MgSO_4$, then to turn, and above 0.1 molar point upwards to the right. At first, therefore, the effect of Mg^{++} predominates over that of $SO_4^=$; but as the concentration of $MgSO_4$ increases the effect of $SO_4^=$ becomes relatively stronger until it predominates and tends to neutralize the initial deviation caused by Mg^{++}.

There is a close similarity between this curve for $MgSO_4$ and the curves of LaMer[3] on higher valence types of other ions, an effect which he attributes to the neglect of higher terms in the Debye-Hückel equation.

We furthermore studied the effect of various proportions of $MgCl_2$ and Na_2SO_4 on oxalate activity. This effect is also shown in Fig. 2 in the lighter curves which form a network. Each curve represents

[3] LaMer, V. K., and Mason, C. F., *J. Am. Chem. Soc.*, 1927, xlix, 410.

either a solution with fixed amount of $MgCl_2$ and varying amounts of Na_2SO_4, or *vice versa*.

It is obvious that we have an antagonism between Mg^{++} ions and $SO_4^{=}$ ions. Presumably this could be formulated in an equation involving both equations (1) and (3) but we have not attempted to do so.

IV.

EXPERIMENTAL.

The experimental procedure is the same as described in the previous paper and requires no amplification. The data are given in Tables II to VI.

TABLE II.

Effect of Na_2SO_4 Alone on pK_2' of Oxalic Acid. (See Fig. 2.)

Calculation of k (where $A = 0$) in 0.01026 molar oxalic acid plus 1.462 equivalents of NaOH plus varying amounts of Na_2SO_4.

Na_2SO_4 added	$\sqrt{\mu}$	pH	δ'	pK_2'	pK_T'	$\Delta_T pK_2'$	f'	k
0	0.142	3.986	1.473	4.030	4.028	+0.002		
0.00417	0.180	3.956	1.474	4.001	3.976	+0.025	1.059	1.8
0.0083	0.235	3.912	1.476	3.954	3.912	0.042	1.102	2.2
0.0167	0.265	3.903	1.476	3.945	3.883	0.062	1.153	2.4
0.0250	0.308	3.873	1.478	3.911	3.844	0.067	1.167	2.1
0.0417	0.381	3.735	1.479	3.871	3.784	0.087	1.222	2.2
0.0833	0.520	3.785	1.481	3.818	3.680	0.138	1.373	2.6
0.167	0.721	3.737	1.483	3.766	3.552	0.214	1.637	3.1
0.250	0.878	3.697	1.485	3.723	3.464	0.259	1.815	3.2
0.333	1.010	3.683	1.485	3.709	3.403	0.306	2.022	3.5
Average.....								2.6

TABLE III.

Antagonistic Action of $SO_4^=$ and Cl^- on pK_2' of Oxalic Acid.

Calculation of k (where $A = 0.015$) in 0.01005 molar oxalic acid plus 1.520 equivalents of NaOH with fixed amounts of Na_2SO_4 and varying amounts of NaCl.

$SO_4^=$	Cl^-	$\sqrt{\mu}$	pH	b'	pK_2'	pK_T'	$\Delta_T pK_2'$	f'	k
0.00833	0	0.212	4.013	1.531	3.960	3.935	0.025	1.059	2.5
"	0.0050	0.214	4.008	1.531	3.955	3.933	0.022	1.052	2.5
"	0.0125	0.240	3.983	1.532	3.928	3.906	0.022	1.052	3.0
"	0.025	0.265	3.956	1.533	3.899	3.884	0.015	1.035	2.6
"	0.050	0.308	3.915	1.534	3.856	3.846	0.010	1.024	2.6
"	0.075	0.346	3.879	1.536	3.816	3.810	0.006	1.014	2.0
"	0.125	0.412	3.829	1.538	3.763	3.758	0.005	1.012	2.5
"	0.250	0.543	3.741	1.542	3.668	3.664	0.004	1.009	3.3
Average									2.6
0.0833	0	0.525	3.853	1.537	3.789	3.676	0.113	1.297	2.3
"	0.0050	0.529	3.868	1.536	3.806	3.672	0.134	1.362	2.8
"	0.0125	0.536	3.859	1.537	3.795	3.667	0.128	1.342	2.8
"	0.025	0.548	3.851	1.537	3.787	3.659	0.128	1.342	2.9
"	0.050	0.570	3.837	1.537	3.773	3.645	0.128	1.342	3.3
"	0.075	0.592	3.783	1.540	3.714	3.631	0.083	1.211	2.3
"	0.125	0.633	3.751	1.543	3.676	3.606	0.070	1.175	2.3
"	0.250	0.725	3.693	1.544	3.617	3.550	0.067	1.167	3.0
"	0.500	0.881	3.602	1.549	3.517	3.464	0.053	1.131	(3.7)
"	0.750	1.012	3.519	1.554	3.425	3.398	0.027	1.064	2.5
Average									2.7
Total average									2.6

TABLE IV.

Action of $SO_4^=$ and Cl^- on 0.005 Molar Oxalic Acid.

1.520 equivalents of NaOH, fixed amounts of Na_2SO_4 and varying amounts of NaCl. $A = 0.36$ in first series and 2.48 in second series.

$SO_4^=$	Cl^-	$\sqrt{\mu}$	pH	b'	pK_2'	pK_T'	$\Delta_T pK_2'$	f'	k
0.00833	0	0.187	4.077	1.522	4.039	3.965	0.074	1.186	92
"	0.0050	0.200	4.064	1.522	4.026	3.952	0.074	1.186	93
"	0.0125	0.218	4.033	1.541	3.962	3.929	0.033	1.080	41
"	0.025	0.245	4.010	1.543	3.935	3.903	0.032	1.077	40
"	0.050	0.291	3.972	1.545	3.893	3.862	0.031	1.074	41
"	0.075	0.332	3.937	1.547	3.856	3.828	0.028	1.067	40
"	0.125	0.400	3.881	1.552	3.790	3.768	0.022	1.052	34
"	0.250	0.534	3.793	1.559	3.690	3.670	0.020	1.047	39
"	0.500	0.732	3.693	1.568	3.573	3.554	0.019	1.045	52
"	0.750	0.866	3.624	1.575	3.492	3.469	0.023	1.055	81
Average									55
0.083	0	0.510	3.937	1.548	3.855	3.685	0.170	1.480	53
"	0.0050	0.515	3.934	1.548	3.852	3.682	0.170	1.480	53
"	0.0125	0.523	3.927	1.548	3.845	3.676	0.169	1.476	53
"	0.025	0.534	3.920	1.549	3.835	3.670	0.165	1.462	51
"	0.050	0.557	3.912	1.549	3.827	3.663	0.164	1.458	51
"	0.075	0.579	3.903	1.550	3.816	3.638	0.178	(1.506)	(57)
"	0.125	0.621	3.867	1.552	3.776	3.613	0.163	1.456	52
"	0.250	0.714	3.810	1.556	3.712	3.555	0.157	1.435	52
"	0.500	0.872	3.727	1.563	3.617	3.468	0.149	1.410	53
"	0.750	1.005	3.676	1.567	3.559	3.405	0.154	(1.425)	(60)
Average									52
Total average									53

TABLE V.

Effect of $MgSO_4$ Alone on the Activity of Oxalic Diion.

0.01026 molar oxalic acid plus 1.462 equivalents of NaOH, plus varying amounts of $MgSO_4$.

$MgSO_4$	$\sqrt{\mu}$	pH	b'	pK_2'	pK_T'	$\Delta_T pK_2'$
0	0.142	3.984	1.473	4.028	4.028	0
0.00313	0.180	3.722	1.483	3.751	3.976	−0.225
0.00625	0.235	3.521	1.496	3.528	3.912	−0.384
0.0125	0.265	3.311	1.518	3.280	3.883	−0.603
0.0188	0.308	3.211	1.533	3.154	3.844	−0.690
0.0313	0.381	3.106	1.554	3.014	3.784	−0.770
0.0625	0.520	3.010	1.577	2.875	3.680	−0.805
0.125	0.721	2.952	1.591	2.791	3.552	−0.761
0.188	0.878	2.939	1.592	2.777	3.464	−0.687
0.250	1.010	2.937	1.588	2.782	3.403	−0.621

TABLE VI.

Antagonism of $MgCl_2$ and Na_2SO_4.

(0.01005 molar oxalic acid, plus 1.520 equivalents of NaOH, with varying amounts of $MgCl_2$ and Na_2SO_4.)

Na_2SO_4	0.00167 M $MgCl_2$				0.00417 M $MgCl_2$				0.00833 M $MgCl_2$			
	pH	b'	pK_2'	$\Delta_T pK_2'$	pH	b'	pK_2'	$\Delta_T pK_2'$	pH	b'	pK_2'	$\Delta_T pK_2'$
0	4.003	1.531	3.950	−0.065	3.896	1.534	3.841	−0.164	3.686	1.543	3.611	−0.381
0.0017	3.994	1.531	3.941	−0.056	3.866	1.535	3.808	−0.179	3.707	1.542	3.634	−0.340
0.0042	3.983	1.532	3.928	−0.040	3.881	1.535	3.820	−0.140	3.714	1.542	3.641	−0.306
0.0083	3.957	1.533	3.900	−0.030	3.866	1.536	3.803	−0.122	3.724	1.542	3.651	−0.263
0.0167	3.937	1.534	3.878	−0.004	3.852	1.537	3.788	−0.089	3.737	1.542	3.664	−0.204
0.0250					3.844	1.537	3.780	−0.058	3.746	1.541	3.675	−0.158
0.0417	3.876	1.536	3.814	+0.030	3.805	1.539	3.757	−0.023	3.744	1.542	3.671	−0.104
0.0833	3.834	1.538	3.768	0.091	3.790	1.539	3.722	+0.046	3.734	1.542	3.661	−0.013
0.167	3.791	1.539	3.723	0.171	3.756	1.541	3.685	0.133	3.724	1.542	3.651	+0.103
0.250					3.735	1.541	3.664	0.200	3.722	1.542	3.649	0.186

V.

SUMMARY.

Sulfate ions ($SO_4^=$) produce an anomalous effect on the ionization of oxalate diion, opposite in direction to the effect of Mg^{++} ions. This effect of sulfate is antagonized by the presence of Cl^- ions according to the equation:

$$k = (f' - 1)\frac{2SO_4^= + Cl^- + A}{(SO_4^=)^{1.5}}$$

where f' is the antilog of the increase in pK_2' due to the sulfate.

In solutions containing up to 0.03 molar $MgSO_4$ the effect of Mg^{++} predominates over that of $SO_4^=$. Above 0.1 molar the effect of $SO_4^=$ predominates and tends to neutralize the initial deviation.

In solutions containing fixed amounts of $MgCl_2$ and varying amounts of $NaSO_4$ (or *vice versa*) the effects of these two salts sharply antagonize each other in all proportions.

THE BRIGHTNESS OF THE LIGHT OF THE WEST INDIAN ELATERID BEETLE, PYROPHORUS.

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(From the Physiological Laboratory, Princeton University, Princeton.)

(Accepted for publication, June 11, 1928.)

The West Indian elaterid beetle, *Pyrophorus*, is one of the brightest of luminous organisms. Pickering, by comparing its light with stars of various magnitudes, believed its intensity to be .004 candle. As Ives has pointed out, it is not so much the intensity, but the specific luminous emission or brightness which is the important quantity in measuring luminescences. The relation between intensity (measured in candles), brightness (measured in lamberts) and specific luminous emission or light flux (measured in lumens) per sq. cm. area of luminescent surface is given by:

$$\frac{\text{candles}}{\text{cm}^2} \times \pi = \frac{\text{lumens}}{\text{cm}^2} \text{ or lamberts.}$$

From Pickering's value, .004 candle, and an assumed light organ area of 1.5 cm.^{2}, which is certainly too large, Ives calculated a specific luminous emission of .0084 lumen per sq. cm. or .0084 lambert brightness. By direct measurement, Ives and Jordan obtained .0144 lambert for the glowworm, and Nichols .016 lambert for luminescence of *Cypridina* (an ostracod crustacean) while Dufford, Nightingale and Calvert found .002 lambert for *Cypridina*.

Thanks to Dr. J. S. Dexter of the University of Porto Rico, Rio Piedras, Porto Rico, and to Mr. R. M. Grey, Soledad, Cienfuegos, Cuba, we have recently received some living *Pyrophorus* beetles (Fig. 1), and have measured the brightness of the prothoracic organ by a modified Macbeth illuminometer. Some of the beetles from Porto Rico were 3 cm. long while those from Cuba were 2.5 cm. or less.

Fig. 2 shows one end of a Macbeth illuminometer. A green filter (*G*) of 24 per cent transmission (measured at the proper color tem-

perature of the illuminometer lamp (I)) is placed before the illuminometer lamp (I) and a neutral filter (N) of 9 per cent transmission before the *Pyrophorus* light organ (P). The green filter allows a good color match, while the neutral filter cuts down the light of *Pyrophorus* to a point where readings can be obtained. In addition, a lens (L_1), placed so as to focus the *Pyrophorus* light at the eye (E), serves to fill the comparison field of the illuminometer, a method suggested by

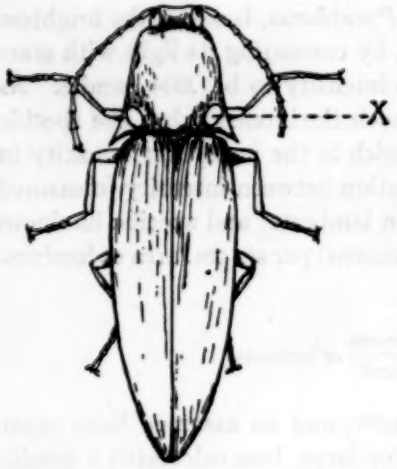

FIG. 1.

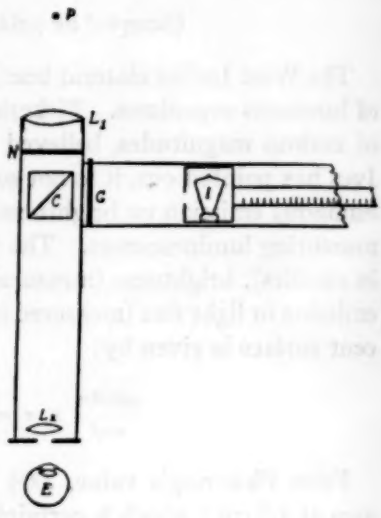

FIG. 2.

FIG. 1. *Pyrophorus* beetle. X, one of the prothoracic light organs.

FIG. 2. P, *Pyrophorus* light organ; N, neutral filter of 9 per cent transmission; G, green glass filter of 24 per cent transmission; C, cube of illuminometer; I, calibrated lamp of illuminometer; L_1, lens which forms a real image of P at eye E; L_2 lens for sharp focussing in illuminometer.

Dr. A. H. Pfund, and serving to make determination of equality in brightness of the two fields easy. About half the measurements were made without the lens, but judgment of equality of brightness is not so easy in this case, as the light organ is small.

When the *Pyrophorus* beetles are squeezed between the fingers, their prothoracic organs glow brilliantly for a considerable period of time, long enough to obtain very good readings. There is much varia-

tion in the brightness of various individuals. About 100 observations on 25 beetles gave values on the inverse square scale of the illuminometer of 4 to 22 foot candles, with an average of 8.5 foot candles. Twenty-two observations on 6 beetles which had been fed sugar water and which gave an unusually bright luminescence, averaged 14.3 foot candles with a maximum of 20 foot candles. Taking a high value frequently observed, 20 foot candles, we calculate the lamberts by making allowance for the screens and the reflection factor of the test-plate used to calibrate the Macbeth illuminometer, as follows:

20 foot candles × .79 (reflection factor*) × 1.076 (conversion factor for foot candles to millilamberts) × 11.11 (reciprocal of .09, the transmission of the neutral filter) × .24 (transmission of the green filter) = 45 millilamberts.

This value may be taken as nearly correct for an active brightly luminescing beetle. It may be used to calculate the candle power of one organ by the relation given above, if we measure the area of a luminous organ. In the beetle giving the greatest brightness the organ was an irregular ellipse with diameters of .147 cm. and .119 cm. The average diameter is .133 cm. and projected area .0137 cm.^{2} Hence .045 lambert or .045 lumen per sq. cm. × .0137 = .00062 lumen. Divided by π lumens per candle this = .0002 candle.

SUMMARY.

The maximum brightness of one of the prothoracic light organs of the West Indian elaterid beetles, *Pyrophorus* (measured at Princeton, New Jersey) was found to be .045 lambert, or .045 lumen per sq. cm. at 20°C. This corresponds to .0002 candle for one organ, or .0004 candle for the pair of prothoracic organs.

* Foot candles is an illumination unit. Multiplied by the conversion factor, 1.076, the illuminated surface would have a brightness expressed in millilamberts, if it reflected 100 per cent of the light. Since no surface does this, the reflection factor (79 per cent) of the particular surface must be used in calculating brightness (millilamberts) when the illuminometer is calibrated in illumination units (foot candles).

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ON HEMOCHROMOGEN.

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(Accepted for publication, June 9, 1928.)

I.

The Nature of Hemochromogen.

Introduction.—In a paper (1) concerned mainly with the rôle of the protein, globin, in determining the fitness of hemoglobin as an oxygen carrier, we presented, in a preliminary way, some observations on the nature of hemochromogen. Since then the study of hemochromogen has assumed an entirely new importance. On the one hand, it has been shown by Keilin (10) that substances related to hemochromogen are present in the aerobic tissues of plants and animals generally. On the other hand—as will appear much more clearly in later papers—hemoglobin has proved to be peculiarly suitable material for the study of the denaturation and coagulation of proteins, and when hemoglobin is denatured in alkaline solution in the presence of a reducer, hemochromogen appears. We have accordingly repeated in greater detail and extended our earlier work.

In hemoglobin the simple protein, globin, is combined, in a manner not yet known, with the iron pyrrol complex, $C_{34}H_{32}N_4O_4Fe$ which we call *heme*. The familiar crystalline substance known as hemin is the hydrochloride of heme. The pigment obtained when a reducing agent such as sodium hydrosulfite ($Na_2S_2O_4$) is added to heme we call *reduced heme*. It is necessary to introduce the word heme in order to be able to refer without confusion to a single substance of definite composition.

It was discovered by Stokes (12) that the addition of alkali and a reducing agent to hemoglobin produces a pigment with a well defined two-banded spectrum whose sharpness greatly impressed the noted physicist. Hoppe-Seyler (9) later gave this pigment the name hemo-

chromogen in order to express the *theory*, which has been generally accepted, that the pigment with the two-banded spectrum is identical with the simple iron pyrrol complex, reduced heme.

We define as a *hemochromogen* a substance containing heme whose spectrum has the characteristic pattern described by Stokes. This definition contains no hypothesis about the nature of hemochromogen or about its relation to reduced heme.

It was noticed over 30 years ago by Bertin-Sans and Moitessier (3) that if pure heme is reduced with sodium hydrosulfite, one does not obtain hemochromogen but that hemochromogen is obtained if one adds further to the solution some protein. No precise suggestion was made about the function of the protein. Despite this work the theory of the identity of reduced heme and hemochromogen continued to be accepted. Sodium hydrosulfite was considered to be an "unsuitable" reducing agent. It was well known that hemochromogen could be obtained from pure heme even without any protein by the use of a "suitable" reducing agent such as hydrazine hydrate. Later Dilling[1] (5) and von Zeynek (13) believed pyridine hemochromogen to contain pyridine in addition to reduced heme. Their notion was that pyridine combined with hemochromogen to give a hemochromogen derivative and that pyridine combined with hemochromogen merely because it was a base.

In this paper we propose to show that reduced heme and hemochromogen are different substances with different properties, and that every hemochromogen consists not simply of reduced heme but of reduced heme combined with some nitrogenous substance.

The Preparation and Properties of Reduced Heme and Hemochromogen.

If pure crystalline hemin is dissolved in $\frac{1}{5}$ N NaOH, in which it is readily soluble, and reduced with sodium hydrosulfite ($Na_2S_2O_4$) there results a sparingly soluble pigment, reduced heme, whose spectrum has a diffuse band in the red. When now an excess is added of any of a great variety of substances which *without exception* contain some

[1] "Hemochromogen acts as an acid and it becomes evident that it might combine with several bases, page 61."

"Pyridine as a base has the power of combining with the double hematin or hemochromogen radicle to form an ester-like substance, page 60."

nitrogen group, two striking changes occur—the precipitate formed by reducing the heme goes into solution, and the spectrum now shows in place of the diffuse band in the red the two sharp characteristic hemochromogen bands first described by Stokes. One can use for the synthesis of hemochromogen from reduced heme proteins like globin or egg albumin, potassium cyanide, hydrazine hydrate, amines, pyridine, pipiridine, ammonia, pyrrol itself, nicotine, and so on. The hemochromogens described in the past were all obtained in the presence of one or more of these substances.[2] The "suitable" reducers such as hydrazine hydrate contained nitrogen, the "unsuitable" ones such as sodium hydrosulfite were nitrogen-free. Although all these hemochromogen-forming substances contain some nitrogen group, not all contain an amino group. On the other hand, not all nitrogen-containing substances yield hemochromogen.

Crystallization.—Some, although not all of the hemochromogens can be crystallized. Dilling (5) found that one can readily obtain a permanent preparation of pyridine hemochromogen crystals by dissolving some hemin in a drop of pyridine on a microscope slide, covering the drop with a cover-slip, and then closing it in with Canada balsam and letting the solution reduce "spontaneously."

We repeated this experiment, using in addition however the reducing agent, sodium hydrosulfite, and obtained the crystals pictured by Dilling.

In the best of such preparations practically all the pigment is in the form of distinct crystals. There is always, however, in addition to the intensely colored crystals a faintly colored background of a very fine precipitate. One cannot see whether this precipitate is crystalline. In order to prove that pyridine hemochromogen can be crystallized it is necessary to show—and this Dilling omitted to do—that the large crystals as well as the fine precipitate give the hemochromogen spectrum, that the crystals are not simply heme whose absorption band is weak.

In the first place, both the crystals and the background are of the same color, a peculiar shade of orange. So they both probably consist of the same substance. We found, furthermore, with the aid of an oil

[2] Dilling did not realize that the various bases contained in his hemochromogens were all nitrogenous substances, or that nitrogen groups play any rôle in hemochromogen formation.

immersion lens and a Zeiss microspectroscope, that a background free of any visible crystals gave a hemochromogen spectrum much less intense than that given by a single group of crystals with a practically colorless background, one actually much less colored than the control background. This indicates that pyridine hemochromogen whatever its precise nature, is a definite crystallizable compound.

Differences between Hemochromogens.

The hemochromogens as a class, then, have certain characteristic properties in common which distinguish them from reduced heme. They are soluble in alkali and have a sharply defined two-banded spectrum, while reduced heme is only slightly soluble in alkali and has a diffuse spectrum. But although all the hemochromogens are similar in a general way, the precise properties of any given hemochromogen depend on what nitrogen substance is used in its preparation. To designate a particular hemochromogen we therefore prefix to the class name the name of the particular nitrogen substance used in its preparation, thus globin hemochromogen or ammonia hemochromogen.

Spectra.—The most convenient and precise although not the only way to distinguish the various hemochromogens is by means of the exact position of the sharp α band. With the aid of the Hartridge reversion spectroscope (6), one can detect and measure differences so small as two Ångstrom units. For example the α band of globin hemochromogen prepared by the addition of $\frac{1}{5}$ N NaOH and $Na_2S_2O_4$ to hemoglobin has its maximum absorption at about 27 Å. u. more to the red than the α band of ammonia hemochromogen.

Different preparations of hemochromogen from the same nitrogen substance always show the α band in exactly the same position. Particularly interesting here is the fact that the hemochromogen prepared from hemoglobin by the addition of alkali and a reducer is spectroscopically indistinguishable from the globin hemochromogen prepared by the addition to an alkaline solution of heme of a reducer and globin prepared by Schulz's method (11).

Color.—Since the individual hemochromogens differ in the positions and intensities of their absorption bands they must differ in color too. These differences are not in all cases apparent to the unaided eye. In

some cases the color difference is striking; for instance, that between pyridine hemochromogen which is somewhat orange and ammonia hemochromogen which is somewhat purplish.

Reduced heme and hemochromogen under the same conditions have very different properties and are, therefore, different substances. Since some second substance is needed for the preparation of hemochromogen from reduced heme and since all that the varied compounds which can be used as this second substance have in common is the possession of some nitrogen group, we conclude that in hemochromogen reduced heme is combined in some manner with a nitrogen group. This combination would account for the change in properties of the pigment when the nitrogen-containing substance is added. The differences in the nitrogenous substances would account for the secondary differences in the various hemochromogens.

Hemochromogen Equilibria.

We shall now describe a few experiments designed to bring out the nature of the reaction between reduced heme and nitrogen groups.

It takes a definite minimum concentration of nitrogenous substance to convert reduced heme into hemochromogen, that is, to produce a soluble pigment whose spectrum shows only the two sharp bands of hemochromogen and none of the characteristic absorption of reduced heme. This minimum concentration varies a great deal from one nitrogen substance to another. In no case is the number of nitrogen groups required less than the number of heme molecules. In some cases it is very much greater. For instance a 3 per cent solution of ammonia is not sufficient to convert reduced heme completely into hemochromogen.

If a solution of ammonia hemochromogen is evacuated, gaseous ammonia is pumped off and the spectrum of reduced heme replaces that of hemochromogen.

If 5 cc. of 28 per cent ammonia is added to 0.1 cc. of a 3 per cent solution in $\frac{1}{5}$ N NaOH of globin hemochromogen prepared from hemoglobin, the spectrum of globin hemochromogen is replaced by that of ammonia hemochromogen whose α band is about 27 Å. u. further towards the blue.

In three ways, therefore, the reaction between reduced heme and

nitrogen compounds is analagous to the reaction between hemoglobin and oxygen or carbon monoxide. It requires a definite concentration of free oxygen or carbon monoxide to give oxyhemoglobin or carbon monoxide hemoglobin. Likewise a definite concentration of nitrogen compound is needed to give hemochromogen. When an oxyhemoglobin solution is evacuated reduced hemoglobin appears. Likewise when an ammonia hemochromogen solution is evacuated, reduced heme appears. When carbon monoxide is bubbled through an oxyhemoglobin solution the spectrum of oxyhemoglobin is replaced by that of carbon monoxide hemoglobin. Likewise when ammonia is added to globin hemochromogen the α band is shifted to the position characteristic of ammonia hemochromogen. We conclude from these facts that just as there is the equilibrium:

$$Hb + O_2 \rightleftarrows Hb\,O_2$$

there is also the equilibrium:

$$\text{Reduced heme} + \text{Nitrogenous substance} \rightleftarrows \text{Hemochromogen}$$

and that hemochromogen is always partially dissociated into its two components, reduced heme and a nitrogen substance. In the case of hemoglobin the lower the temperature the more the equilibrium is shifted to the right in favor of oxyhemoglobin. We shall see that the same holds true in the case of the hemochromogen equilibrium.

As has just been described, if enough ammonia is added to globin hemochromogen the spectrum of globin hemochromogen is replaced by that of ammonia hemochromogen. If, however, less ammonia is added then the α band of the resulting solution is intermediate between the α band of globin hemochromogen and that of ammonia hemochromogen. The more ammonia is added, the nearer is the band to that of ammonia hemochromogen. These intermediate spectra can be imitated by means of optical mixtures of the spectra of the two hemochromogens such as are observed by looking at the same time through cells containing separate solutions of the two hemochromogens.

The mere fact that the α band of the solution becomes indistinguishable from that of NH_3 hemochromogen when enough ammonia has been added does not mean that all the globin hemochromogen has

disappeared. A small amount of globin hemochromogen cannot be detected spectroscopically in the presence of sufficient ammonia hemochromogen.

II.

The Reactions between Reduced Heme and Cyanide.

We shall now discuss the reactions of a particular nitrogenous substance, cyanide, with reduced heme. These reactions have a peculiar interest because due to cyanide's great affinity for reduced heme these reactions provide a means of determining the composition of cyan-hemochromogen and because cyanide is able in small concentration to inhibit tissue respiration.

The Two Cyanide Compounds.

Two distinct pigments can be obtained by adding cyanide to reduced heme (1) cyan-hemochromogen which has not hitherto been described and (2) what will be provisionally called the second cyanide compound of reduced heme. This non-commital name is given because the nature of the compound is not yet certain. If the concentration of cyanide is low enough one gets only cyan-hemochromogen, if high enough only the second compound. Intermediate concentrations of cyanide give mixtures of the two pigments. Cyan-hemochromogen has two typical hemochromogen bands designated α and β. The α band is much more intense than the β and has its maximum absorption at about 5535 Å. u. The spectrum of the second compound likewise has two sharp bands, in this case of about equal intensity. The α band is about 150 Å. u. toward the red of the corresponding band of cyan-hemochromogen. In mixtures of the two pigments the two α bands do not fuse. From the relative intensities of these two bands one can, therefore, obtain a rough idea of the relative concentrations of the two components of the mixture.

The Composition of Cyan-Hemochromogen.

The difficulty of isolating hemochromogen free of uncombined reduced heme and nitrogenous compound led to an attempt to determine the composition of hemochromogen by an indirect method. The following experiments are designed to determine how much cyanide

has to be added to reduced heme to convert it into hemochromogen, that is, to cause the complete replacement of the characteristic spectrum of reduced heme by that of hemochromogen. Four solutions of 2×10^{-4} M hemin in 1 per cent Na_2CO_3* are made up to contain .65, .75, 1, and 1.25 molecules of KCN per molecule of heme. After addition of the reducer $Na_2S_2O_4$, layers 1.5 cm. thick are examined spectroscopically. Solution .65 shows the α band of cyan-hemochromogen and marked reduced heme absorption in the yellow; solution .75 more hemochromogen and less reduced heme; and solution 1.25 no reduced heme but in addition to cyan-hemochromogen a small amount of the second cyanide compound. Since one molecule of cyanide is adequate to convert one molecule of heme into hemochromogen, cyan-hemochromogen cannot contain more than one cyanide group per heme.

The following control experiment shows that were 25 per cent of the pigment in solution 1 reduced heme, the reduced heme would be detectable spectroscopically, despite the presence of the hemochromogen. Solution 1 contains 2×10^{-4} M reduced heme in 1 per cent Na_2CO_3 plus 2×10^{-4} M KCN. Another solution is made up which contains $.5 \times 10^{-4}$ M or 25 per cent as much reduced heme and no cyanide. The two solutions, each 1.5 cm. thick, are placed together in front of the spectroscope and observed at the same time. One can distinctly see reduced heme in addition to the hemochromogen.

The Affinity of Reduced Heme for Cyanide.

The experiments which have just been described do not exclude the possibility that cyan-hemochromogen contains less than one cyanide group per heme for we have shown that hemochromogen is always partially dissociated into and in equilibrium with its two components, reduced heme and the nitrogenous substance. In the case of the typical hemochromogen, cyan-hemochromogen, there is the equilibrium mixture.

$$\text{Cyan-hemochromogen} \rightleftarrows \text{Reduced heme} + \text{Cyanide}.$$

*Na_2CO_3 was used instead of NaOH because, as R. Hill (7) has pointed out, the precipitation of reduced heme is slower in Na_2CO_3 than in NaOH. Our original experiments with NaOH, however, gave essentially the same results.

Thus if a solution of cyan-hemochromogen containing just enough cyanide to cause the disappearance of the reduced heme bands is warmed or diluted, the characteristic absorption of reduced heme reappears. In solution 1, therefore, part of the cyanide is combined with reduced heme as cyan-hemochromogen and part is free serving to drive the equilibrium to the left, to prevent much dissociation of the hemochromogen. If the concentration of free cyanide is small compared with 2×10^{-4} M, that is, if the affinity of reduced heme for cyanide is very great, then the free cyanide in solution 1 can be neglected and cyan-hemochromogen contains neither more nor less than one cyanide per heme. If, however, the free cyanide is not negligible, then cyan-hemochromogen contains less than one cyanide per heme.

Let us assume, although there is as yet no experimental basis for this assumption, that the concentration of free cyanide needed to convert a given fraction of the heme into hemochromogen is independent of the total concentration of the pigment. For any given value of the fraction $\frac{\text{Reduced heme}}{\text{Cyan-hemochromogen}}$ the lower the total concentration of pigment, the lower the concentration of cyan-hemochromogen or of bound cyanide. The concentration of free cyanide, however, according to our assumption is always the same. Therefore, the lower the total concentration of pigment the greater is the free cyanide relative to the bound. By lowering the pigment concentration enough one can make the free cyanide practically equal to the total cyanide added. This condition is met in the following experiments.

A solution is made up in 1 per cent Na_2CO_3, 3.75×10^{-6} M in respect to heme and 3.75×10^{-5} M, or 10 times more concentrated, in respect to cyanide. The pigment is reduced with $Na_2S_2O_4$. From the previous experiments the molarity of the bound cyanide is at most equal to the heme with which it is combined, that is at most 3.75×10^{-6} M and, therefore, less than 10 per cent of the total cyanide. The solution is examined spectroscopically through a polarimeter tube 20 cm. long. The intensity of the bands is about the same as that of a more concentrated solution in a narrower vessel. One can see only cyan-hemochromogen—no reduced heme and none of the second cyanide compound. If the cyanide concentration is doubled the

intensity of the α band of the hemochromogen is not visibly increased. If the cyanide concentration is halved, the α band is less intense and one can see reduced heme. To prevent any visible dissociation of cyan-hemochromogen then, when the cyan-hemochromogen concentration is 3.75×10^{-6} N in respect to iron, the concentration of free cyanide must be about 3.75×10^{-6} N.

To discover what differences in cyan-hemochromogen concentration are detectable, three solutions of cyan-hemochromogen are made up in 1 per cent Na_2CO_3. In all three cases the cyanide concentration is $2 \times 3.75 \times 10^{-6}$ N, that is more than adequate to convert reduced heme into hemochromogen. The heme concentrations are 3.75×10^{-6} N, 25 per cent more, and 25 per cent less. In none of the solutions can either reduced heme or the second compound be seen. The differences in the intensities of the cyan-hemochromogen bands, however, are distinct. Thus in the experiments already described, the formation of 25 per cent additional hemochromogen on doubling the cyanide concentration would be detectable.

The assumption has been made that the cyanide concentration needed to give a definite value of the fraction $\frac{\text{Reduced heme}}{\text{Cyan-hemochromogen}}$ is independent of the total pigment concentration. The percentage of reduced heme which is detectable spectroscopically is likewise roughly independent of the pigment concentration for a decrease in the pigment concentration is compensated for by an increase in the thickness of the layer examined. It follows that the concentration of free cyanide needed to prevent visible dissociation of cyan-hemochromogen is the same, namely about 3.75×10^{-5}, when the total heme concentration (reduced heme plus hemochromogen) is 2×10^{-4} M as in the first experiments as it is found experimentally to be when the total heme concentration is only 3.75×10^{-6} M.

When the total heme concentration is 2×10^{-4} M however, in order to prevent visible dissociation of the hemochromogen, the total cyanide concentration must be 2×10^{-4} M. If the free cyanide is only 3.75×10^{-5} or about $\frac{1}{5}$ of the total cyanide then it is within the rather large experimental error and can be neglected. The bound cyanide can be set equal to the total cyanide.

In brief the experiments with the relatively concentrated heme show

that cyan-hemochromogen does not contain more than one cyanide per heme. The experiments with the relatively dilute heme show that reduced heme in alkaline solution has a high affinity for cyanide, and taken together with the other experiments they indicate, although they do not prove conclusively, that cyan-hemochromogen does not contain less than one cyanide per heme.

The Second Cyanide Compound.

The solution containing 1.25 molecules of cyanide per molecule of heme shows in addition to cyan-hemochromogen the second compound. As the cyanide concentration is increased one sees more and more of the second compound and less cyan-hemochromogen. Finally the reduced heme is converted completely into the second compound and the addition of more cyanide causes no further change in the spectrum. Experiments similar to those with cyan-hemochromogen show that the concentration of free cyanide needed to convert reduced heme completely into the second cyanide compound is some 25 times as great as is needed to prevent the dissociation of cyan-hemochromogen.

The factors which determine the composition of the equilibrium mixture:

$$\text{Second compound} \rightleftarrows \text{Cn Hc} \rightleftarrows \text{CN} + \text{Reduced heme}$$

are the cyanide concentration, the total heme concentration, and the temperature, and the hydrogen ion concentration. The importance of this last factor will be discussed in a later paper. Thus in a mixture of the two cyanide derivatives, more of the second compound appears and less cyan-hemochromogen if first, more cyanide is added; second, the absolute amounts of cyanide and heme being kept constant, the concentrations of both are increased by using less water; and third, the solution is cooled.

The method used to determine the composition of cyan-hemochromogen is likewise applicable to the second compound. Much more free cyanide, however, is needed to prevent its dissociation than the dissociation of cyan-hemochromogen. In order that the free cyanide be negligible therefore in comparison with the bound cyanide, the total pigment concentration must be much greater than is the case with cyan-hemochromogen. In the concentrated solution it is difficult to

determine spectroscopically the precise number of cyanide molecules needed to convert a molecule of reduced heme completely into the second compound. We are prepared to state only that the number is a small one.

Our experiments to determine the relations of the second cyanide compound to other hemochromogens and to carbon monoxide hemochromogen will not be described here because they do not seem capable at present of any simple explanation. This applies especially to experiments in which cyanide is added to reduced heme in the presence of various concentrations of other nitrogen substances.

This investigation of the reactions of cyanide with reduced heme in alkaline solution has shown, so far as we know for the first time, that there are two distinct cyanide derivatives of reduced heme; that one is a typical hemochromogen in which cyanide behaves like a typical nitrogen substance; that reduced heme has a great affinity for cyanide; and that cyan-hemochromogen probably contains one cyanide group per heme.

There have been two attempts to determine the composition of pyridine hemochromogen. The first was made by von Zeynek (13) before the nature of hemochromogen was understood. He found solid pyridine hemochromogen to contain 2.2 molecules of pyridine per molecule of heme. Von Zeynek regarded his single experiment as only preliminary and gave no conclusive evidence that the solid analyzed was pure pyridine hemochromogen. Recently R. Hill (7) has concluded that pyridine hemochromogen contains two pyridine groups per heme. This conclusion is hardly justified by the experiments given and the soundness of the experiments we regard as questionable. Hill's work will be discussed in detail in a separate paper.

III.

The Reactions between Reduced Heme and Proteins.[3]

We shall now consider the reactions between reduced heme and the most complicated of the hemochromogen-forming substances, the proteins. The complication consists in the fact that the individual

[3] These experiments were described in the doctoral dissertations of the authors which were submitted to Cambridge University in the spring of 1926.

protein molecule contains a large number of free nitrogen groups. One cannot know in advance which and how many of these groups combine with reduced heme. These protein reactions are of special interest because it is by combination with a particular protein, native globin, to form hemoglobin that heme acquires the variety of properties which permit it to act as an oxygen carrier.

Globin.

The first experiments are an attempt to find out how much heme a given amount of globin can convert into hemochromogen. More and more heme is added to the protein until there appears in addition to the hemochromogen bands, the characteristic absorption of reduced heme.

A stock solution of globin hemochromogen is prepared by adding to 1 cc. of 3 per cent horse hemoglobin, 2 cc. of $\frac{1}{3}$ N HCL (which denatures the protein) and then adding NaOH. From this there is prepared a series of solutions all $\frac{1}{10}$ N in respect to NaOH, all containing .033 per cent of globin hemochromogen from hemoglobin, and finally containing in addition varying amounts of extra hemin. On the assumption that the molecular weight of globin is 16,700, the minimum molecular weight, the solutions contain 1, 2.5, 5, 7.5, 10, and 25 molecules of heme per molecule of globin. The solutions are reduced with $Na_2S_2O_4$ and examined spectroscopically.

The differences in the intensities of the hemochromogen bands are striking. The more heme in the solution, the darker and broader the hemochromogen bands. Only in solution 25 can any reduced heme at all be detected. The following experiments show that such results are not given by optical mixtures of reduced heme and hemochromogen such as are observed by looking through two cells at the same time which contain separate solutions of hemochromogen and reduced heme.

Cell 1 contains globin hemochromogen in the same concentration as in the experiments just described, and cells 2 and 3 contain reduced heme, the concentration in respect to heme being 2.5 times greater in cell 2 and 5 times greater in cell 3 than in the hemochromogen solution. Looking through the hemochromogen solution and cell 2

at the same time one can just distinguish reduced heme. When cell 3 is used instead of cell 2 the reduced heme absorption is marked. These experiments show that in the original experiments where reduced heme is added to globin hemochromogen and not kept in a separate cell there did not result a simple mixture of hemochromogen and reduced hemochromogen. One can detect reduced heme in such mixtures.

The fact that the original solution 10 shows no reduced heme and much more hemochromogen than solution 1 therefore indicates that a single molecule of denatured globin can convert at least 10 molecules of heme into hemochromogen.

On adding extra heme to globin hemochromogen two changes can be noted besides the increase in the intensity of the hemochromogen band. Solutions 10 and 25 are hazy and there is a shift of the α band to the red which in solution 25 amounts to about 10 Å. u. The haziness may be due to a decrease in the solubility of the molecule when the globin is loaded with heme; the shift of the band either to a change in the chemical composition of the pigment or in its dispersion.

Since the individual globin molecule seems to have a considerable number of nitrogen groups which can react with reduced heme to form hemochromogen, there must result theoretically, when denatured globin is added to reduced heme, a complicated equilibrium in which a number of nitrogen groups compete for the heme. Practically, however, this complexity is often of no great consequence. The hemochromogen prepared from hemoglobin is of constant composition and is not detectably dissociated into reduced heme at all, any more than acetic acid in strongly acid solution is dissociated—except to a minute theoretical extent—into acetate ions.

Whether extra heme hemoglobin corresponding to the artifical globin hemochromogens which contain extra heme can be prepared is, in the absence of experiments, an open question. Hill and Holden (8) estimate native globin by its oxyhemoglobin-forming capacity. This procedure depends on the tacit and unproven assumption that the composition of the synthetic hemoglobin is independent of the concentration of heme during the synthesis.

Edestin and Zein.

All proteins have not the same hemochromogen-forming capacity. Thus we have found that it takes much more edestin than globin to convert a given amount of heme into hemochromogen and more zein than edestin. These differences are not surprising. We have already seen from a study of the reactions between reduced heme and the simpler nitrogen compounds that the amount of hemochromogen present at equilibrium—the heme concentration and the temperature being kept constant—depends on the number and the nature of the nitrogen groups. It requires a minimum amount either of cyanide or ammonia to convert a given amount of reduced heme into hemochromogen, but it requires much less cyanide than ammonia. In the case of the different proteins the nature of the nitrogen groups and their number per gram of protein varies a great deal. It is not surprising that accompanying these differences in the number and nature of the nitrogen groups, proteins have differences in hemochromogen-forming capacity. The low hemochromogen-forming capacity of zein may be connected with its low diamino acid content (4).

The Difference between Globin Hemochromogen and Hemoglobin.

Based on the classical theory that hemochromogen and reduced heme are identical is the further theory which, so far as we know, has never been questioned that when hemoglobin is converted into hemochromogen the protein, globin, is split off from the iron pyrrol complex, heme. We have shown, however, that globin hemochromogen like hemoglobin itself is a compound of globin and heme. The question thus arises, as to what the difference is between hemoglobin and the hemochromogen obtained from it. We have already (2) pointed out one difference, namely that hemochromogen is a compound of heme and denatured globin while hemoglobin is a compound of native globin. The evidence given for this was that hemoglobin is a typical coagulable protein, and that the denaturation of hemoglobin in alkaline solution as tested for by solubility always runs parallel with formation of hemochromogen as tested for spectroscopically. There may be still other differences between hemoglobin and globin hemochromogen. We do not know whether these two substances have the same molecular weight or whether heme and globin are combined in the same way

in hemochromogen as in hemoglobin, or in precisely what way they are joined together in either compound.

Hill and Holden (8) have confirmed our view that globin hemochromogen is a denatured protein without seeing fit to mention our paper.

CONCLUSIONS.

1. Every hemochromogen consists of the iron pyrrol complex, reduced heme, combined with some nitrogenous substance.

2. In every hemochromogen there is the equilibrium:

$$\text{Hemochromogen} \rightleftarrows \text{Reduced heme} + \text{Nitrogenous substance.}$$

3. Cyanide can form two distinct compounds with reduced heme, one of which is the typical hemochromogen, cyan-hemochromogen.

4. Reduced heme in alkaline solution has a great affinity for cyanide.

5. Cyan-hemochromogen probably contains one cyanide group per heme.

6. The hemochromogen prepared from hemoglobin is a compound of denatured globin and reduced heme.

7. The individual molecule of denatured globin, of hypothetical molecular weight 16,700, can convert at least 10 molecules of reduced heme into hemochromogen.

8. The hemochromogen-forming capacity of globin is, under given conditions, greater than that of edestin, which in turn, is greater than that of zein.

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SYNERESIS AND SWELLING OF GELATIN.

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The name syneresis was given by Graham[1] in 1864 to the phenomenon of the breaking up of jellies on long standing or when disturbed. The jelly then, instead of consisting of one homogeneous mass, becomes segregated into solid lumps surrounded by a thin liquid. Syneresis is quite common in jellies or, as they are called, gels of gelatin, agar, starch, etc. It is possible that the separation of serum from clotted blood and the splitting of soured milk into curd and whey may also be classified under the same term. Various theories have been suggested as to the possible cause of syneresis.

Thus Ostwald[2] considers syneresis as simply a separation of phases similar to the separation of phases in a critical fluid mixture. Lloyd[3] explains syneresis of isoelectric gelatin as being due to the absence of soluble gelatin salts, which otherwise keep the network extended by their osmotic pressure. As it will be seen from further discussion, Miss Lloyd's hypothesis is partly true with respect to the absence of syneresis in gelatin in the presence of electrolytes, although there is apparently some confusion as to the meaning of "soluble gelatin salts." With the exception of some recent work by Liepatoff[4] on *geranine* and Mukoyama[5] on *viscose*, very little quantitative work has been done on syneresis in general and of gelatin in particular.

The writer approached the problem of syneresis in gelatin through

[1] Graham, T., Chemical and physical researches (collected papers), Edinburgh 1876, 619.

[2] Ostwald, W., An introduction to theoretical and applied colloid chemistry, New York, 1917, 93.

[3] Lloyd, D. J., *Biochem. J.*, 1920, xiv, 165.

[4] Liepatoff, S., *Kolloid.-Z.*, 1927, xliii, 396.

[5] Mukoyama, T., *Kolloid.-Z.*, 1927, xlii, 79.

the study of the swelling of blocks of dilute gelatin when immersed in water. All of the former studies on swelling of gelatin have dealt with the swelling of dry gelatin or of concentrated gels. In all these cases there is always a gain of water and an increase in size of the gelatin block even at low temperatures. Quite different results are obtained when blocks of isoelectric gelatin of concentrations of less then 10 per cent are immersed in cold water. Instead of gaining water the blocks of gelatin lose water, and the lower the concentration of gelatin the greater is the amount of water lost.

Table I shows the values of swelling at 5°C. of gelatin gels of various concentrations when immersed in M/1000 acetate buffer pH 4.7. The

TABLE I.

Swelling of Blocks of Gels of Various Gelatin Content at 5°C. in M/1000 Acetate Buffer pH 4.7.

Electrolyte-free isoelectric gelatin has been used in making up the gels. Gels set for 24 hrs. at 5°C. before they were placed in the buffer solution.

Concentration of gelatin in gm. per 100 cc. solution.....	3.0	4.0	5.0	6.0	8.0	10.0	12.0	14.0	16.0	20.0
Original weight of block of gel (after setting).....	1.610	1.600	1.505	1.790	1.890	1.847	2.020	2.120	2.090	2.250
Final weight of block at equilibrium (after 20–25 days).....	1.150	1.140	1.130	1.435	1.730	1.845	2.200	2.470	2.570	3.050
Per cent change in weight.	−29	−29	−25	−20	−8	0	+9	+17	+23	+36

values are those obtained at equilibrium, which is reached after 20 to 25 days. It is seen that while at concentrations above 10 per cent the gelatin blocks gain in weight, at the lower concentrations there is a continuous drop in weight.

The theories of swelling as developed by Procter, Wilson, Loeb, Northrop and the writer deal with the process of positive swelling of gelatin. These may be summarized as follows:

1. Swelling is mainly a process of osmosis due to a greater concentration of mobile molecules in the block over that of the outside solution in which it is immersed.

2. The molecules causing osmosis may be diffusible ions, as in the case of swelling due to acids, alkalies and some salts, or non-diffusible molecules or groups of molecules as in the swelling of isoelectric gelatin.

3. A block of solid gel behaves like a true elastic body. The swelling is regulated by the elasticity of the gelatin block. At equilibrium the elastic pressure is equal and opposite to the osmotic pressure.

4. When a gelatin sol sets to a gel it is under no elastic strain as long as it is not immersed in water. As soon as it is put into water or salt solution the osmotic forces begin to act against the elasticity of the block causing a strain in the block due to an increase in the bulk. A strain is also brought about in a block of gelatin when water is removed from it by evaporation.

The tendency of a block of a dilute gel to shrink when placed in distilled water indicates that there is still a possible strain in the gel while it sets, which is contrary to the apparent non-strained condition of gels of higher concentration, as demonstrated by Northrop.[6] With the object of finding a possible explanation of this peculiar behavior of dilute gels the writer undertook a detailed study of the "negative swelling" of gelatin under various conditions.

Experimental Procedure.

All the experiments as well as the weighings were done in a refrigerator room kept at a temperature of about 5°C.

Solutions containing various amounts of isoelectric gelatin in distilled water of pH 4.7 or in salt solutions were heated to 50°C. and coated on weighed microscopic slides (1.5 cc. per slide), or 2 cc. were poured into moulds, consisting of short Pyrex glass tubing of about 15 mm. diameter, which were mounted on a clean paraffin block. The gelatin was allowed to set in the refrigerator for about 20 hours. Care was taken to prevent evaporation by keeping the slides in "moist chambers" or stoppering the tubes with rubber stoppers. After the period assigned for setting, the slides or the blocks, on removal from the tubes, were weighed and put into 150 cc. M/1000 acetate buffer pH 4.7 or into other solutions as described later. After various intervals of time the slides or blocks were dried with filter paper and weighed. In drying blocks of dilute gelatin it was found more convenient to use a clean towel instead of filter paper.

The fluid originally used as the outside solution was distilled water. This was brought to pH 4.7 by means of acetic acid.* But it was soon found that the pH of the water increased slightly after a few days, with the result that the gelatin began

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[6] Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 901.

* Distilled water made up with acetic acid to pH 4.7 is designated throughout this paper as H_2O pH 4.7.

to gain in weight instead of reaching a constant value. Hence distilled water was replaced by M/1000 acetate buffer pH 4.7.

Fig. 1 shows the effect of two concentrations of acetate buffer pH 4.7 as well as of H_2O on the negative swelling of 4 per cent isoelectric gelatin coated on slides. During the first few days there was a loss of water in all the gelatin blocks; but afterwards those that were kept in

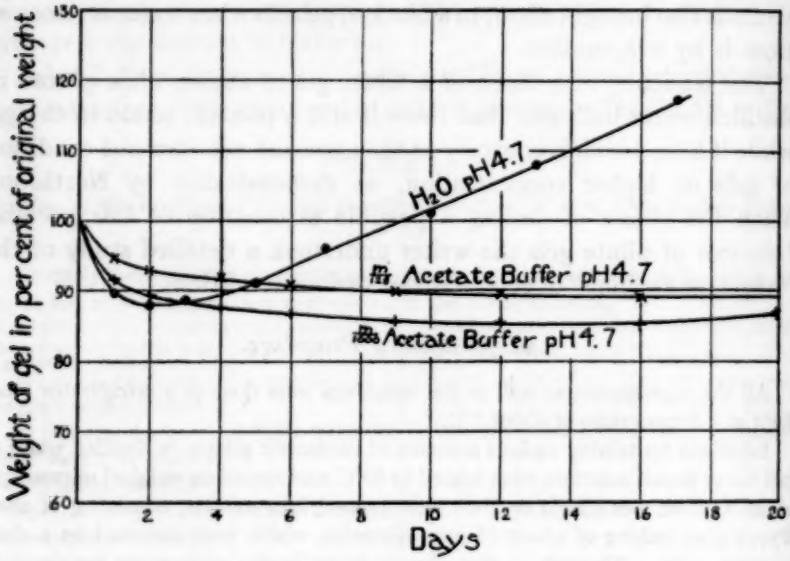

FIG. 1. Effect of a slight change with time in the pH of the outside solution on the change in weight of 4 per cent gels at 5°C. The pH of the distilled water became slightly higher than 4.7 on standing.

H_2O began to gain weight continuously, while the others that were put in buffer solution kept on losing weight until the 10th day when an approximate equilibrium was established. After the 20th day the block in M/1000 buffer began to gain slightly in weight but on renewal of the buffer the equilibrium weight was reached again. The experiment also shows that there is less loss in weight of gelatin with the increase in concentration of salt in the surrounding medium. This is simply due to the fact that salts generally increase the swelling of isoelectric gelatin up to a concentration of M/2.[7]

[7] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–28, viii, 317.

Is the Negative Swelling Due to Solution of the Gelatin?

A qualitative test on the protein content of the surrounding medium showed that only a trace of the gelatin goes into solution. Quantitative experiments were carried out as follows:

Blocks of 4 per cent of isoelectric gelatin were allowed to remain in large volumes of M/1000 acetate buffer pH 4.7 until the loss in weight became constant. The blocks were then removed, put into weighed evaporating dishes, weighed and then dried in an electric oven at 100°C. for 24 hours. In all cases the dry weight measurements checked with the weight of gelatin as calculated from the loss of

TABLE II.

Effect of Duration of Setting on the Negative Swelling of 5 Per Cent Isoelectric Gelatin in M/1000 Acetate Buffer pH 4.7.

Time of setting	0	16 hrs.	40 hrs.	68 hrs.	92 hrs.	6 days	8 days
Weight of gelatin after being in moist chamber, in per cent of original weight	100	99.4	100	101	98.5	95	100
Final weight of gelatin in M/1000 acetate buffer at equilibrium	87.0	91.0	90.0	86.0	91.0	88.4	90.0

water. Thus it is clear that while some of the gelatin may go into solution, the amount is too insignificant to affect the results. The negative swelling is due to loss of pure solvent.

Effect of Duration of Setting on the Negative Swelling of Gels.

A number of weighed slides were coated with 5 per cent isoelectric gelatin. The slides were then reweighed and placed over water in rubber-stoppered glass tubes. Care was taken that the gelatin was not in contact with the water. After various intervals of time the slides were removed, weighed and put into M/1000 buffer pH 4.7. The results are given in Table II.

It is seen that the duration of setting has very little effect on the final loss of water from the gel. In addition the experiment shows that the amount of water lost from the gelatin while kept in a closed moist chamber is very small during the first few days.

Effect of Drying of Gels.

A series of slides were coated with 5 per cent isoelectric gelatin solution and then placed for various lengths of time in loosely covered Petri dishes the cover of which had a padding of moist filter paper. This was done in order to prevent too rapid drying of the gels. The slides were afterwards put into M/1000 buffer pH 4.7. Table III shows that the final loss of water is independent of the loss of water due to evaporation. If more water was lost by evaporation than would have been the case had the block been immersed in water, then the gelatin gains water when placed in liquid. In other words, the equilibrium value can be obtained from two directions.

TABLE III.

Effect of Drying on the Equilibrium of Negative Swelling of 5 Per Cent Isoelectric Gelatin in M/1000 Acetate Buffer pH 4.7.

Time of drying, in hrs.	4	16	44	68	92	116
Weight of gelatin after drying, in per cent of original weight	99.0	97.0	93.0	90.0	87.0	83.0
Final weight of gelatin in M/1000 acetate buffer	87.0	87.5	87.5	87.5	88.0	87.0

Effect of Volume of Outside Solution or Size of Block on the Equilibrium Value of Negative Swelling.

A 4 per cent solution of isoelectric gelatin was allowed to set for several days in a stoppered flask. No fluid was observed on the surface of the gelatin except for a few drops of condensed vapor on the sides of the flask. A lump of about 10 gm. of gel was then removed by means of a spatula and placed in a weighed porcelain Gooch crucible and its weight determined. The gel was then mashed up by means of the spatula. Fluid immediately began to ooze from the gelatin. Also fluid appeared in the cavity in the stock of gelatin in the flask. The crucible was left to drain overnight on a Gooch funnel. The weight of the gelatin next morning was found to be 82 per cent of the original. The crucible was then placed in a beaker with enough M/1000 acetate buffer to reach the edge of the crucible (about 50 cc.). After several

hours the weight of the gelatin became about 80 per cent and next day it reached the value of 77 per cent, which then became constant. Its dry weight was then determined and was found to check with the weight as calculated from the loss of water. The amount of water lost here by the 4 per cent gel through "syneresis" was identical with the amount of water lost by a solid block of 4 per cent gel weighing 1.5 gm. and immersed in 150 cc. of liquid with several changes of the outside solution. In this last case it took about 10 days to reach the equilibrium state. In another experiment two solid blocks of 4 per cent gel each weighing about 3.5 gm. were placed in stoppered Pyrex tubes, to one of which was added about 5 cc. of H_2O pH 4.7, while the other tube had no water at all. But the pressure of the block of gelatin on the glass was enough to start the diffusion of fluid from the gelatin with the result that the loss in weight of both blocks was identical, each losing 17 per cent in weight after 3 days.

In complete absence of water the process of syneresis is very slow due to the slow rate of diffusion of water through the dry surface of the solid gelatin. A trace of water placed on the surface through actual addition or through pressure is enough to start a rapid diffusion of the water from the solid gelatin. A similar occurrence takes place when a water-permeable collodion bag is filled with water and a moderate pressure applied to it. The rate of diffusion of water from the bag is much greater when the bag is immersed in water than when the bag is placed in a moist chamber after it has been dried carefully with a towel. In an actual experiment the writer found that while it took only about 1 minute for a definite volume of water under a pressure of 10 cm. mercury to diffuse out from a collodion bag when immersed in water, it required about 20 minutes for the same volume of water under the same pressure to diffuse out from the same bag after it was dried outside with a towel and placed in a closed tube *over* water. When the bag was immersed in water again the diffusion became as rapid as before.

The Mechanism of Syneresis.

The experiments thus far described prove that syneresis of gelatin can be conveniently studied by measuring the loss of water in dilute gelatin blocks when kept in dilute buffer pH 4.7 at low temperature,

since the same quantity of water is lost finally whether the block is immersed in a large volume of water or when syneresis is initiated through mechanical forces such as shaking, pressure, etc. Fig. 2 shows the rate of gaining or losing water by isoelectric gels of various gelatin content when kept in M/1000 acetate buffer pH 4.7 at 5°C. The equilibrium values of the concentrations of gelatin in the various gels were plotted separately in Fig. 3 against the original concentra-

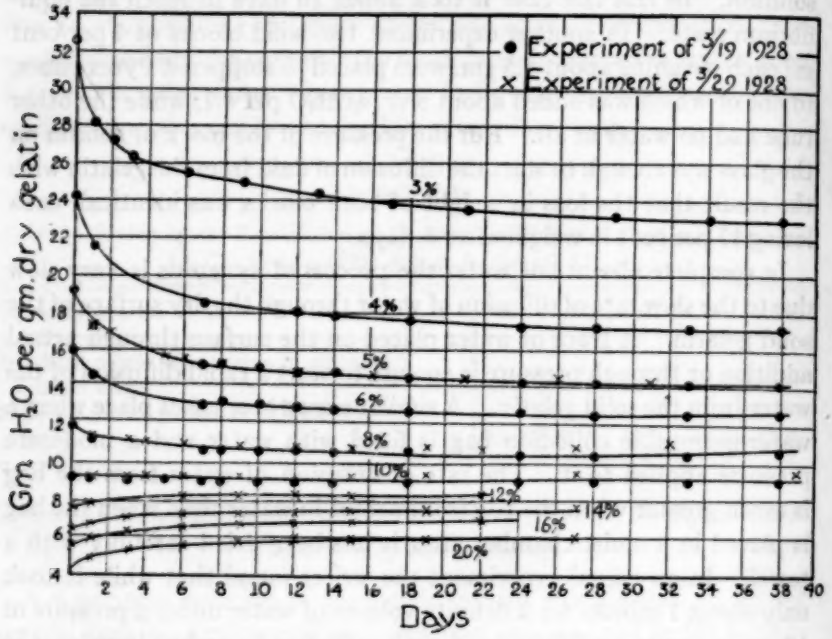

FIG. 2. Swelling of various concentrations of isoelectric gelatin blocks in M/1000 acetate buffer pH 4.7 at 5°C. The gelatin was allowed to set for 24 hours at 5°C. before placing it in the buffer solution.

tions. It is seen that all the points lie on a smooth curve, no matter whether there has been positive or negative swelling, thus suggesting that there is a common mechanism to both forms of swelling. The theoretical relation between the original and final concentrations of a swelling gel has been developed by Northrop[8] for concentrations of

[8] Northrop,[6] p. 898.

gelatin above 10 per cent. His theory is based on the assumption that a block of gelatin behaves like a perfect elastic body, namely that in accordance with Hooke's law any strain in the block is proportional to the stress producing it. A block of freshly set gel is under no strain

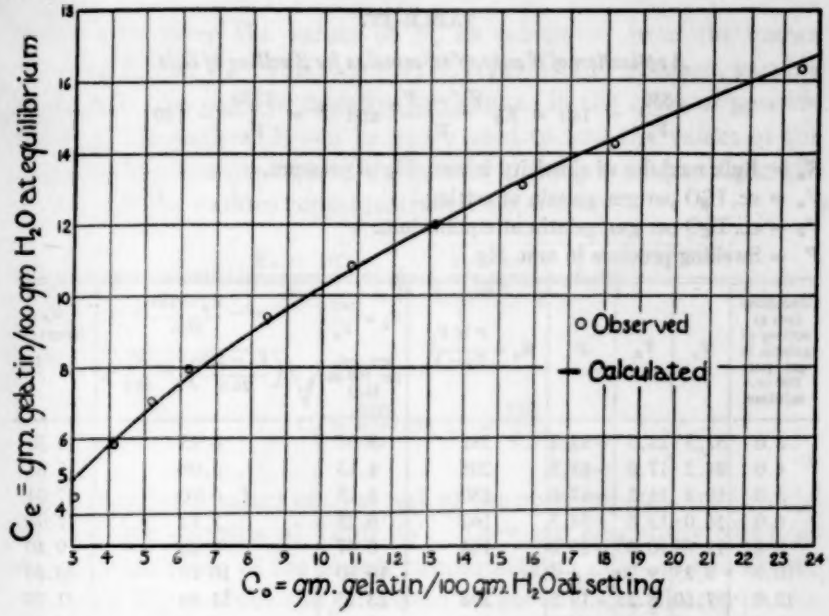

FIG. 3. Relation between the original and the final concentrations of the gelatin in swelling of gels. The dots are the experimental values. The smooth curve represents the theoretical relation:

$$C_e = -\frac{K_e - 140}{26.6} + \sqrt{\left(\frac{K_e - 140}{26.6}\right)^2 + \frac{K_e}{13.3} C_o}$$

where $K_e = 195$.

but as soon as it is placed in water the block swells under the influence of a certain swelling pressure P and a strain is produced in the block. At equilibrium we have

$$P = K_e \frac{V_o - V_e}{V_e} \qquad (1)$$

Where K_e is bulk modulus of elasticity of the block and V_o and V_e are cubic centimeters of water per gram of dry gelatin at setting and at equilibrium respectively.

The swelling pressures of gels of higher than 10 per cent have been

TABLE IV.

Application of Northrop's Formulas for Swelling of Gels.

$$\frac{1330}{V_e} - 140 = K_e \frac{V_e - V_o}{V_o} \text{ and } P = \frac{1330}{V_e} - 140$$

K_e = Bulk modulus of elasticity in mm. Hg of pressure.
V_o = cc. H_2O per gm. gelatin at setting.
V_e = cc. H_2O per gm. gelatin at equilibrium.
P = Swelling pressure in mm. Hg.

Concentration at setting of gelatin in gm. per 100 cc. solution	V_o	V_e	P	$K_e = \frac{P \times V_o}{V_e - V_o}$	$C_o = \frac{100}{V_o}$ gm. gel. per 100 cc. H_2O	$C_e = -\frac{K_e - 140}{26.6} + \sqrt{\left(\frac{K_e - 140}{26.6}\right)^2 + \frac{K_e}{13.3} C_o}$	C_e observed $= \frac{100}{V}$
3.0	32.5	23.0	−82.2	281	3.07	4.95	4.35
4.0	24.2	17.0	−61.8	208	4.13	5.98	5.88
5.0	19.2	14.2	−47.0	180	5.18	6.90	7.04
6.0	16.0	12.6	−34.5	163	6.25	7.72	7.94
8.0	11.80	10.65	−16.0	164	8.47	9.26	9.40
10.0	9.25	9.20	+4.5		10.80	10.70	10.86
12.0	7.60	8.35	+19.5	198	13.15	11.98	11.98
14.0	6.42	7.60	+35.0	191	15.6	13.20	13.15
16.0	5.50	7.00	+50.0	183	18.2	14.40	14.30
20.0	4.26	6.10	+78.0	181	23.5	16.60	16.40
Average.....				195			

measured directly and found that they can be expressed empirically as the following function of V_e, namely,

$$P = \frac{1330}{V_e} - 140 \tag{2}$$

Combining the two equations we get the following relation between V_e and V_e:

$$\frac{1330}{V_e} - 140 = K_e \frac{V_e - V_o}{V_o} \tag{3}$$

As shown by Northrop, this relation holds well for the swelling of blocks of gels of a gelatin content of more than 10 gm. per 100 cc. H_2O. The same formula was applied by the writer to the cases of negative swelling of gels of lower than 10 per cent with the following results.

Table IV gives the values of K_c as calculated from the known values of V_c and V_o. It is seen that K_c is practically constant, varying as much in the cases of negative swelling as in the cases of positive swelling. Equation (3) can be easily used to find the values of the equilibrium concentrations of the gelatin in the gels in grams per 100 cc. H_2O for the various concentrations used, namely,

$$C_c = -\frac{K_c - 140}{26.6} + \sqrt{\left(\frac{K_c - 140}{26.6}\right)^2 + \frac{K_c}{13.3} C_o}$$

where

$$C_c = \frac{100}{V_c} \text{ and } C_o = \frac{100}{V_o}$$

The calculated values of C_c as well as the observed ones are given in the last two columns of Table IV. They are practically identical with the exception of the lowest concentration. This is also shown clearly on Fig. 3 where the calculated values of C_c are plotted on the smooth curve.

Thus it is evident that the same laws which hold for swelling of gels of a gelatin content higher than 10 gm. per 100 cc. H_2O hold also for the process of giving off water by gels of a lower gelatin content. The active force P in both processes can be calculated by means of equation (1). The values for P are positive in the high concentrations and negative in the lower concentrations, all lying on one straight line which crosses the zero axis at a concentration of gelatin of about 10 per cent (see Fig. 4).

What is Swelling Pressure?

It has been assumed by Northrop and the writer[9] that the active force P, *i.e.*, the swelling pressure in gels, is brought about by the

[9] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 162.

osmotic pressure of the soluble ingredient in the gel, and is numerically equal to it. But the fact that the swelling pressure becomes negative in gels of a gelatin content of less than 10 per cent indicates that osmotic pressure is not the only factor which causes swelling, that there is also another force which evidently works in the opposite

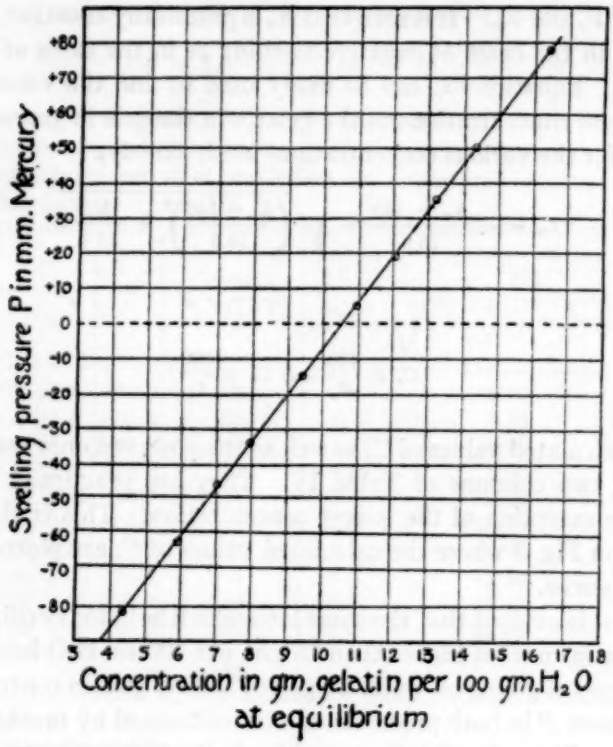

FIG. 4. Swelling pressure of gels of various gelatin content pH 4.7 at 5°C., from the relation $P = 13.3\,C_g - 140$.

direction to that of the osmotic force, and that the observed swelling pressure is the resultant of the two oppositely acting forces. It will be shown in the following discussion that the force that causes shrinking of dilute gels is connected with the micellar structure of gelatin solutions and is brought about by the strain prevalent in the micellæ before the gelatin has set to a gel.

Loeb[10] first established the theory that the increase of hydration of gelatin in solution due to a change in pH, as shown by viscosity measurements, is brought about by the swelling of the micellæ in the gelatin solution. The micellæ swell because of a higher osmotic pressure inside of the micellæ than outside due to a difference in the ion distribution in accordance with the law of "Donnan equilibrium." The writer[11] has further developed Loeb's idea of hydration by osmosis by applying it to the case of hydration of gelatin at its isoelectric point, where in the absence of diffusible ions no Donnan equilibrium occurs. It was, namely, shown that the hydration of isoelectric gelatin in solution, as demonstrated by viscosity and osmotic pressure measurements, is caused by the fact that each micella in gelatin consists of an insoluble shell containing a definite amount of a soluble ingredient of gelatin. The latter exerts an osmotic pressure on the micella and brings about an inflow of water into it until the osmotic pressure in the micella is balanced by the total osmotic pressure of the solution acting against it and its elastic pressure. The equilibrium state can be expressed as $P_i - P_o = Eq$ where P_i and P_o are the osmotic pressures inside and outside of the micella, respectively, E is a constant proportional to the bulk modulus of elasticity of the micella, and q is the amount of water of hydration per gram of gelatin. At low concentration of gelatin the outside osmotic pressure is small, hence the micellæ take up individually relatively large amounts of water. But as the total concentration increases the opposing outside osmotic pressure increases and the micellæ swell less, with the result that q, *i.e.* the amount of hydration per gram of gelatin gradually becomes less and less. Thus, although the micellæ are at equilibrium with the outside solution, they are still under a strain exerted by a pressure equal to Eq, the magnitude of which decreases with the increase in the total concentration of gelatin.

The Theory of the Rôle of the Micellæ in Swelling of Gelatin.

The hydrated micellæ in a gelatin solution are kinetically free and are able to exert osmotic pressure as any other particles or molecules.

[10] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924, 270.

[11] Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 811.

The first effect of cooling of a gelatin solution is a decrease in the kinetic energy of the micellæ. Their motion becomes sluggish with more chance to adhere to each other, until they begin to form fibrils. Gradually the fibrils interlace and form a network occluding the rest of the gelatin solution. In the absence of electrolytes most of the soluble gelatin becomes insoluble or precipitates out as soon as the temperature has fallen low enough to make the solution supersaturated with respect to it. The micellæ which were under a strain before the gelatin began to set, due to the osmotic pressure of the soluble ingredient inside of the micellæ, now shrink and lose water owing to the precipitation of the soluble gelatin inside of the micellæ. This loss of water probably takes place even before the gelatin becomes solid, but as soon as it solidifies the shrinkage of the micellæ becomes slower because of the resistance to the diffusion of the liberated water offered by the dry surface of the gel, as mentioned before. But as soon as the gel is brought in contact with water a rapid diffusion of water from the block of gel takes place. Each individual micella shrinks, with the result that the whole network contracts and it expels not only the water that was inside of the micellæ but also a great deal of water that was held by capillarity between the micellæ. The shrinkage of the micellæ continues until it is balanced by the elastic resistance of the block as a whole.

Effect of Concentration of Gelatin.

As stated before, the strain due to the inner osmotic pressure in each individual micella in a gelatin solution becomes less as the total concentration of the gelatin increases. Hence when the stress on the micella is removed by the "precipitation" of the soluble gelatin there is less contraction in the micellar network of concentrated gels than in the dilute ones, and less syneresis. There is also another factor which makes the syneresis decrease with the increase in the concentration of the gel, namely the osmotic pressure of the gel as a whole. It was shown by Northrop and the writer[12] that even at a temperature as low as 0°C. there is still enough soluble material left in a solid gel to produce osmotic pressure. The amount of soluble material naturally

[12] Northrop and Kunitz,[9] p. 174.

increases with the increase in total concentration of gelatin. Hence concentrated gels not only do not lose water but actually gain water. Thus syneresis and swelling are caused by two opposite forces acting against the elasticity of the solid block of gel, namely the elastic strain in the micellæ and the osmotic pressure of the block as a whole. At low concentration the first one is prevalent, at higher concentrations the second force becomes more important; while at a concentration of

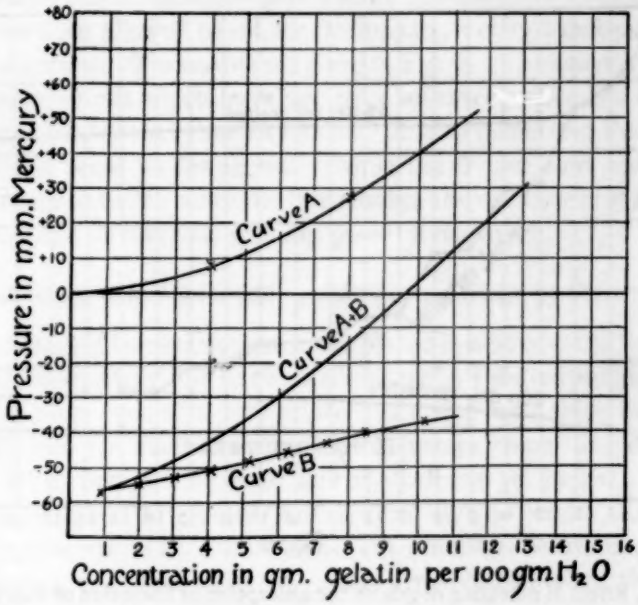

FIG. 5. Curves: *A* for osmotic pressure of isoelectric gelatin solutions at 25°C., and *B* for the stress on the micellæ at 35°C. The resultant curve $A + B$ represents the hypothetical curve for swelling pressure of gels.

about 10 per cent the two forces balance each other and the block of gel neither swells nor loses water. The observed swelling pressure P is then the algebraic sum of the two forces and it is balanced by the elastic pressure of the block of gel.

There seems to be no way to determine directly either the osmotic pressure of a gel or the elastic strain in the micellæ of a gelatin solution at its setting point. Still, an idea of the character of the curve

representing the sum of the two pressures at various concentrations of gelatin may be obtained by plotting the observed values for osmotic pressure of various concentrations of gelatin solutions at a temperature above the setting point, and also the values for the elastic strain in the micellæ as obtained from measurements of viscosity of gelatin solutions. Fig. 5 shows the curve for osmotic pressure at 25°C.[13] plotted as positive, and the curve for *Eq* (strain in the micellæ) at 35°C. [14] as

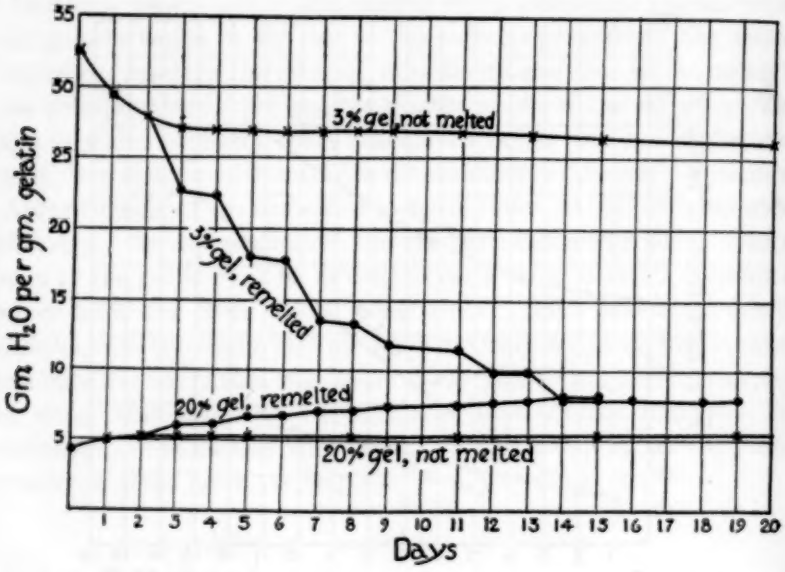

FIG. 6. Effect of reheating of gels on the absorption or liberation of water at 5°C.

negative values of *P*. The third curve represents the algebraic sum of the two pressures. It resembles closely the actual curve for swelling pressure as shown in Fig. 4.

Effect of Reheating of Gels.

The limiting factor in swelling or in shrinking of gels is the elasticity of the block. When the block of gel is heated the elastic strain caused by the swelling is released. Hence when the gel is cooled again and

[13] Northrop and Kunitz,[9] p. 166.
[14] Kunitz, [11] p. 820.

put back into water the gel continues swelling until the swelling pressure is balanced by the new elastic pressure. This has been shown by Northrop[15] to hold true for positive swelling. The writer tried the effect of reheating on two gels, one of which was of 3 per cent gelatin content and the other of 20 per cent content. For this purpose the gelatin solutions were coated on slides, allowed to set for 24 hours, weighed and put into M/1000 acetate buffer pH 4.7. Every 3rd day the gels were heated carefully over a small alcohol flame until melted. They were then allowed to set for 30 minutes, weighed and put back into the solution. The results are given in Fig. 6. The smooth curves are those of blocks which have not been reheated, while the broken curves are those of the reheated blocks. It is to be noticed that the 3 per cent gel keeps on losing water while the 20 per cent continues gaining water until the concentration reaches about 12 per cent in both cases, where no further change takes place on reheating.

Effect of pH of the Gelatin on the Loss of Water by Gels.

When blocks of isoelectric gelatin are placed in acid or alkali solution they swell enormously due to the setting up of a Donnan equilibrium. This takes place even with very dilute gels. The same thing happens when the gels are made up of gelatin solutions containing acid or alkali. When put into water or acid or alkali the gel swells. It has also been observed by Jordis[16] and noticed by the writer that gels containing electrolytes are quite stable even in dilute solution, and no syneresis occurs. Quite different results are obtained when the acid or alkali is removed by dialysis.

Experiment.—A series of solutions of 3 per cent gelatin were made containing various amounts of HCl or NaOH. The solutions were heated to 50°C., then poured into tubes (2 cc. in each) and allowed to set in a refrigerating room for 24 hours. The blocks were afterwards removed from the tubes, weighed and put into 150 cc. M/30 acetate buffer pH 4.7 where they were kept for several days until the weight became constant. The gels were then transferred to M/1000 acetate buffer pH 4.7 and kept there until new equilibrium had been

[15] Northrop.[6]

[16] Jordis, E., *Z. Electrochem.*, 1902, viii, 677.

established. The M/1000 buffer was renewed several times during the experiment.

The results are given in Fig. 7. It is seen that gels which had a pH other than that of the isoelectric point of gelatin lose more water than gels made of isoelectric gelatin. This shrinking takes place only after the acid or alkali has been removed by neutralization and dialysis. The greater shrinking of gels which contained at setting some acid or

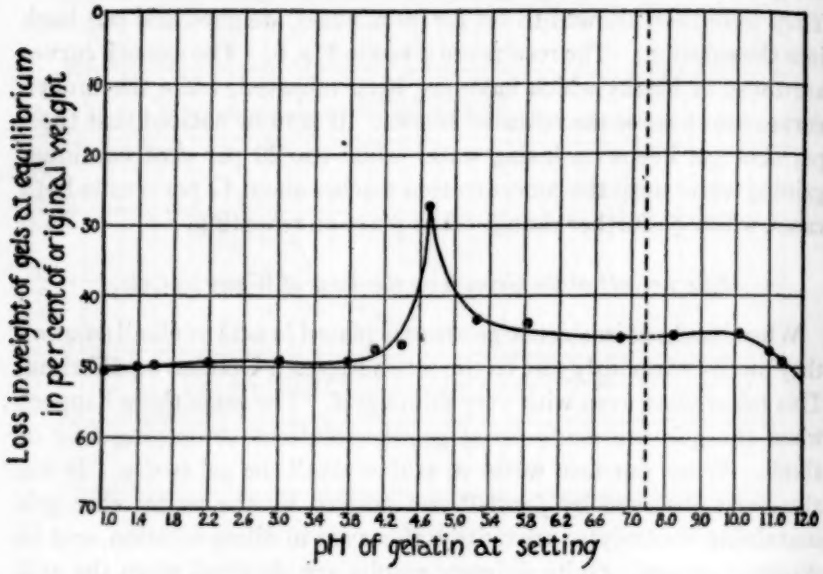

FIG. 7. Effect of pH on syneresis of 3 per cent gel at 5°C.

alkali in solution over those which were electrolyte-free takes place only in gels of low gelatin content. The difference disappears in case of gels of higher concentrations. This is shown in Table V. At a concentration above 6 per cent the loss in water is identical for all gels no matter whether they originally contained acid or alkali or were made up of pure isoelectric gelatin. The peculiar effect of the pH of the original solutions on the loss of water by the gels after the gel was brought back to the isoelectric point becomes clear in view of the theory developed here.

The Theory of Setting of Gels Containing Acid or Alkali.

In dilute solutions of gelatin containing moderate amounts of acid or alkali the micellæ are much more swollen than at the isoelectric point due to a greater concentration of diffusible ions inside than outside of the micellæ. Cooling and setting does not change this unequal distribution of ions. Hence, until the ions are removed by dialysis the micellæ do not lose water and no syneresis takes place. But as soon as the acid or alkali is removed by neutralization and dialysis the micellæ shrink much more than in the case of originally isoelectric

TABLE V.

Swelling of Various Concentrations of Gelatin of Various pH in M/30 Acetate Buffer pH 4.7 at 5°C.

Expressed as per cent of original weight.

Concentration of gelatin in gm. per 100 cc. solution.....		3	4	5	6	8	10
1928							
2/15	pH 9.0. 15 days in M/30 acetate	75	84		99	110	118
2/24	" 4.7. 15 " " " "	95	96		103	108	114
3/10	" 4.7. 2 " " " "	97	98	100	101	106	110
3/10	" 2.0. 2 " " " "	83	90	95	101	106	111
3/10	" 4.7 2 " " " " then 8 days in H_2O pH 4.7	79	85	87	88	95	102
3/10	" 2.0 2 days in M/30 acetate then 8 days in H_2O pH 4.7	57	70	77	85	93.3	101

gels. With increase in the concentration of gelatin the pH effect on the swelling of the micellæ in a gelatin solution diminishes rapidly, as shown by viscosity measurements,[17] with the result that the pH effect on syneresis in the solid gels is also diminished with increase in concentration of the gelatin. There is also another factor in the effect of acid or alkali on syneresis, namely the solubility effect which is shown also by salts.

The Effect of Salts on Syneresis.

It is generally known that salts affect the solubility of gelatin and the rate of setting (Levites[18]). At temperatures above the setting point of

[17] Kunitz,[11] p. 832.

[18] Levites, S. J., *Z. Chem. u. Ind. Kolloid.*, 1907, ii, 161.

solutions of gelatin salts split the micellæ into smaller units thus giving rise to higher osmotic pressures (Northrop and Kunitz[19]). There is very little action of the salts on the soluble fraction of gelatin, which is in solution even in the absence of salts. The total volume of the micellæ is not changed to any considerable extent, and the viscosity of the gelatin solutions is only slightly increased by addition of salt. As a salt-free dilute solution of isoelectric gelatin is allowed to set the soluble fraction both inside and outside of the micellæ gradually

TABLE VI.

Effect of NaCl on Swelling of 3 Per Cent Gelatin Gels.

Measured after 24 hours in solution.

	Weight in per cent of original weight of block.							
Concentration of NaCl pH 4.7.....	0 (H_2O)	M/256	M/128	M/64	M/32	M/16	M/8	M/4
I. 3 per cent salt-free isoelectric gelatin blocks put into salt solutions	88.6	94.0	95.4		100	102	104	108
II. 3 per cent isoelectric gelatin blocks made up in varying concentrations of NaCl and put into the corresponding salt solutions	86.6	88.5	87.4	89.4	91.2	93.2	94.6	98.4
III. 3 per cent isoelectric gelatin blocks made up in various concentrations of NaCl and put into H_2O pH 4.7	90.0	86.0	84.0	82.0	80.0	77.0	73.0	
IV. 3 per cent isoelectric gelatin blocks made up in various concentrations of NaCl. Kept in M/1000 acetate buffer pH 4.7 until equilibrium was established, and then weighed	71.0		60.0	56.0	55.0	54.0		50.0

"precipitates" out during the setting. As stated before, the micellæ lose some of their water of hydration during setting, and the block of gel begins to lose weight almost immediately after it sets if the conditions for diffusion of the water are favorable.

In the presence of salts the setting is slower, first because of the finer state of the micellæ, and secondly because the salts prevent the

[19] Northrop and Kunitz,[7] p. 332.

precipitation of the "soluble" ingredient of gelatin, as shown by higher swelling of isoelectric gelatin in the presence of salts. The micellæ lose much less water during setting than in the absence of salts. It is possible that the micellæ even take up water during setting in the presence of salts because of the reduction of the outside osmotic pressure due to the immobilization of the micellæ.

When the salt-containing gel is put in H_2O pH 4.7 or dilute buffer of the same pH, the salt dialyzes out and the soluble gelatin both inside and outside precipitates out as in case of originally salt-free gels. The stress on the micellæ due to the greater concentration of soluble

TABLE VII.

Effect of M/8 *NaCl on Swelling of Concentrated Gels of Isoelectric Gelatin at 5°C.*

Gels were made up in M/8 NaCl, allowed to set for 24 hrs. and then put into M/1000 acetate buffer pH 4.7. Buffer changed several times.

Concentration of gelatin in gm. per 100 cc. solution.....	10	12	14	16	20
Cc. H_2O per gm. of gelatin at setting.....	9.20	7.55	6.37	5.48	4.23
" " " " " " " equilibrium....	9.20	8.30	7.50	6.90	6.00
Per cent increase.....	0	+10.0	+17.6	+26.0	+42.0
Same gels but without salt					
Cc. H_2O per gm. gelatin at setting.....	9.25	7.60	6.42	5.50	4.26
" " " " " " equilibrium	9.38	8.35	7.60	7.00	6.10
Per cent increase.....	+1.5	+10.0	+18.5	+27.5	+43.0

gelatin inside than outside is removed and the micellæ shrink, thus bringing about a greater loss of water due to syneresis in salt-containing gels than in salt-free gels of pH 4.7, as shown in Table VI. It is interesting to observe that the salt-containing gel, which unlike the salt-free gel is not turbid in appearance, does not assume any turbidity even after the salt is dialyzed out. On the other hand when a milky white salt-free gel is placed in salt solution or acid the gel swells but the turbidity persists, thus proving the turbidity is caused by the structure of the micellæ before setting. The turbidity is apparently caused only by coarse micellæ. The splitting of micellæ into finer elements by salts prevents turbidity formation.

Is the Effect of Salt on Syneresis Due to a Change in the Bulk Modulus of Elasticity of the Block?

A possible explanation of the peculiar effect of dilute salt solutions on syneresis is that the elasticity of the gel is affected by the presence of salt in it and that even after removal of the salt from the gelatin by dialysis the elasticity of the block still remains altered. The change expected would be a decrease in the bulk modulus so that a smaller

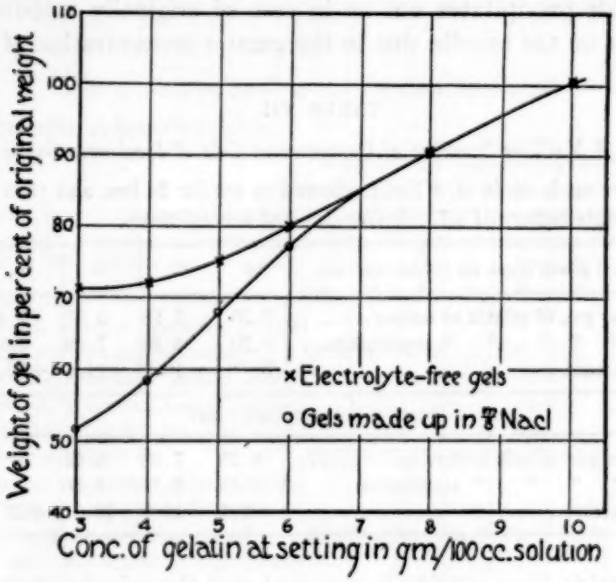

FIG. 8. Effect of salt on syneresis of various concentrations of gelatin blocks in M/1000 acetate buffer pH 4.7 at 5°C.

force is required to compress the block. But if salts in low concentrations affect the elasticity of a gel then it is to be expected in gels of high gelatin content, that those which originally contained salt and from which the salt was afterwards removed would swell more than gels made up of ordinary isoelectric gelatin. Table VII shows that this is not the case. The swelling of gels of a gelatin content of more than 10 per cent is identical whether the gels originally contained salt or not. The effect of salt is only on the shrinking of gels of low concen-

trations, and it disappears at a concentration of about 8 per cent, as shown in Fig. 8, and is best explainable by the effect of salts on the solubility of the gelatin in the micellæ.

The Solubility Effect of Dilute Acid or Alkali on Syneresis.

Acids and alkalies in addition to their pH effect on gelatin have also an enormous effect on the solubility of gelatin. This explains why the curve for syneresis effect of pH does not give a maximum point similar to the pH viscosity curves. A continuous increase in acid or alkali concentration has the same effect on the solubility of gelatin as addition of large amounts of salt and at higher concentrations of acid or alkali the solubility effect prevails.

SUMMARY.

1. When solid blocks of isoelectric gelatin are placed in cold distilled water or dilute buffer of pH 4.7, only those of a gelatin content of more than 10 per cent swell, while those of a lower gelatin content not only do not swell but actually lose water.

2. The final quantity of water lost by blocks of dilute gelatin is the same whether the block is immersed in a large volume of water or whether syneresis has been initiated in the gel through mechanical forces such as shaking, pressure, etc., even in the absence of any outside liquid, thus showing that syneresis is identical with the process of negative swelling of dilute gels when placed in cold water, and may be used as a convenient term for it.

3. Acid- or alkali-containing gels give rise to greater syneresis than isoelectric gels, after the acid or alkali has been removed by dialysis.

4. Salt-containing gels show greater syneresis than salt-free gels of the same pH, after the salt has been washed away.

5. The acid and alkali and also the salt effect on syneresis of gels disappears at a gelatin concentration above 8 per cent.

6. The striking similarity in the behavior of gels with respect to syneresis and of gelatin solutions with respect to viscosity suggests the probability that both are due to the same mechanism, namely the mechanism of hydration of the micellæ in gelatin by means of osmosis as brought about either by diffusible ions, as in the presence of acid or

alkali, or by the soluble gelatin present in the micellæ. The greater the pressures that caused swelling of the micellæ while the gelatin was in the sol state, the greater is the loss of water from the gels when the pressures are removed.

7. A quantitative study of the loss of water by dilute gels of various gelatin content shows that the same laws which have been found by Northrop to hold for the swelling of gels of high concentrations apply also to the process of losing water by dilute gels, *i.e.* to the process of syneresis. The general behavior is well represented by the equations:

$$P_1 - P_2 = K_e \frac{V_e - V_0}{V_e}$$

and

$$P_1 - P_2 = \frac{1330}{V_e} - 140$$

where P_1 = osmotic pressure of the soluble gelatin in the gel, P_2 = stress on the micellæ in the gelatin solution before setting, K_e = bulk modulus of elasticity, V_0 = volume of water per gram of dry gelatin at setting and V_e = volume of water per gram of gelatin at equilibrium.

The writer wishes to express his indebtedness to Dr. John H. Northrop for very valuable suggestions and advice.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION ON THE RATE OF HYDROLYSIS OF GLYCYL GLYCINE, GLYCYL LEUCINE, GLYCYL ALANINE, GLYCYL ASPARAGINE, GLYCYL ASPARTIC ACID, AND BIURET BASE BY EREPSIN.

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INTRODUCTION.

The characteristic effect of the hydrogen ion concentration on the activity of erepsin was explained by Euler (1) on the assumption that the enzyme attacked only the ionized form of the substrate. Michaelis (2), on the other hand, has ascribed the effect of the hydrogen ion concentration on the activity of several other enzymes to changes in the ionic species of the enzyme. One of the writers (3) found in the case of pepsin or trypsin that the pH-activity curve varied with different proteins and that the general shape of the curves could be predicted by assuming that pepsin acted upon the positive protein ion and trypsin on the negative protein ion, without making any assumptions in regard to the ionization of the enzyme. The hydrolysis of dipeptides by erepsin furnishes a favorable test of these assumptions since the equilibria governing the substrate are better known than is the case with most enzyme reactions. If the effect of pH depends primarily on changes in the condition of the enzyme, the pH-activity curves for various dipeptides should be the same, while if the pH effect is primarily on the substrate then the pH-activity curves for peptides having different titration curves should be different and should vary with the titration curves of the peptides. The rate of hydrolysis of glycyl glycine (GG), glycyl alanine (GA), glycyl leucine (GL), glycyl asparagine ($GApNH_2$), glycyl aspartic acid (GAp) and biuret base (B) at various pH values has been determined under

conditions such that the relative rate of hydrolysis (at the different pH values) was independent (or nearly so) of the enzyme concentration and of the substrate concentration. It was found that the pH-activity curve shifts with the titration curve of the substrate so that the condition of the substrate is evidently one of the determining factors. The equilibrium conditions in the substrate, however, are not sufficient in general to enable the entire pH-activity curve to be calculated and it is necessary to assume in addition that the enzyme is a weak acid or base with a dissociation constant of about $10^{-7.6}$ (*i.e.*, $pK_e' = 7.6$). Independent evidence for this assumption has been found in the effect of the pH on the rate of destruction of the enzyme and the effect of neutral salts on the rate of the reaction. If it is then assumed that the reaction takes place between the ionic forms given in Table I the pH-activity curves may be calculated with a fair degree of accuracy.

TABLE I.

The Ionic Species of Substrate and Enzyme between Which Reaction May Take Place.

	I. Low pH form of substrate acted on by high pH form of enzyme		II. High pH form of substrate acted on by low pH form of enzyme	
Substrate	Peptides	Biuret base	Peptides	Biuret base
Substrate charge	Zwitterion ±	Cation +	Anions − or −− Zwitteranion* ±−	Non-ionized 0
Enzyme charge if an acid	Anion (−)		Non-ionized (0)	
if a base	Non-ionized (0)		Cation (+)	

* The dianion (−−) and zwitheranion (±−) forms refer only to glycyl aspartic acid (of the substances under discussion).

Experimental Methods.

Preparation of Erepsin Solution.—Fresh small intestine of swine was thoroughly washed and run through a chopper several times. The resulting thick paste was mixed with an equal volume of glycerin and toluene was added. It was allowed to stand at 17–20°C. for 3 days and then filtered through cheese-cloth. The resulting solution keeps indefinitely at 0°C. Before use the solution was further

purified by dialysis in a strong, tightly stoppered collodion sac against cold running tap water for 24 hours. A heavy flocculent precipitate forms during this time and is filtered off. The filtrate is again dialyzed for 24 hours against cold tap water and again filtered. This filtrate is used in a final dilution of about 1 to 10. In this dilution the formol titration is negligible and the hydrolysis of 0.02 M GG is half completed in about 2 hours. Dry weight determinations on this solution give a few per cent residue which, however, seems to consist largely of glycerin so that the value is of no significance. The solution has no action on gelatin.

Preparation of the Dipeptides.—The dipeptides, except biuret base, were prepared from racemic amino acids according to Fischer (4) and were recrystallized until the melting points and amino nitrogen figures were correct.

The biuret base was prepared according to Curtius (5). The substance obtained, however, was insoluble in chloroform but in other respects agreed with that described by Curtius. When dissolved in water it gave a solution of about pH 8.0 and when titrated with acid and alkali a titration curve having a pK value of about 8.3 was obtained. On complete hydrolysis with erepsin the equivalent of 3 carboxyl groups were liberated so that it was assumed that the substance was a mixture of the free base and the carbonate. Lack of material prevented more definite identification.

Method of Following the Reaction.—In order to determine the pH-activity curve it is necessary to carry out the reaction at constant pH. The pH may be kept constant by the use of phosphate buffers or by using only the first part of the curve. In the latter case the peptides serve as the buffer if in addition M/100 Na acetate is added to buffer the range from 5.0 to 7.0. Both methods were used at first and it was found with glycyl glycine that the results were the same. The presence of phosphate retards the reaction and as it was later found necessary to work with as dilute erepsin as possible the later experiments were done in the absence of phosphate and the reaction followed only until 20 per cent complete. The pH was measured electrometrically before and after the experiment and was found not to vary sufficiently to affect the results.

Method of Titration. Phosphate Present.—Sufficiently large samples were taken, depending on the concentration of the peptide, to give an initial formol titration of 2 to 5 cc. 0.01 M NaOH. 1 cc. 10 per cent ferric chloride solution was added and sufficient 0.2 M NaOH to make the solution alkaline to phenolphthalein. The phosphate, iron and any protein present were thus precipitated and filtered off. The filtrate was brought to pH 5, 1 cc. formalin added and the solution titrated as previously described (6).

In the absence of phosphate the formalin was added and the sample titrated direct. All titrations were made in triplicate and usually agreed within 0.05 cc. 0.01 M NaOH. Six to ten complete series of hydrolysis at intervals of about 0.5 pH from 5 to 9 were run on each peptide.

Temperature.—All experiments, except those on the rate of inactivation of the enzyme, were made at 25°C.

Method of Calculation, etc.—According to the simple theory of a catalytic reaction the course of the reaction is predicted by the monomolecular formula and the velocity constant obtained in this way should be independent of the substrate concentration and proportional to the catalyst concentration. In the case of enzyme reactions this is not usually the case, the velocity constants decrease as a rule as the substrate concentration increases. If this effect of the substrate concentration on the velocity constant depended on the pH of the solution it is evident that different pH-activity curves would be obtained depending on the concentration of substrate. To be significant the curves must depend only on the pH and be independent of the substrate concentration. It was found in the case of glycyl leucine that the velocity constants decreased much more rapidly with increasing substrate concentration at pH 8.5 than at pH 6.0 so that the relative rate of hydrolysis at pH 8.5 compared to pH 6.0 decreased with increasing glycyl leucine concentration. In order to avoid this difficulty it was necessary to carry

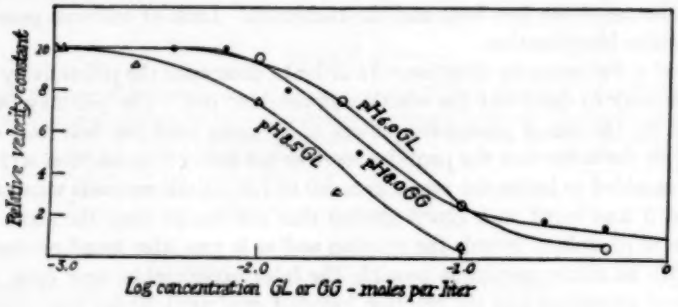

FIG. 1. Effect of concentration of glycyl leucine and glycyl glycine on velocity constant at various pH values.

out the experiments at as low a concentration as possible. The experimental limit for the methods used is about 0.01 M and this concentration was used throughout. In the case of glycyl glycine the effect of concentration was less marked. The results of the experiments on this point are shown in Fig. 1.

Enzyme Concentration.—von Euler and Josephson have noted that the inhibitory effect of glycine on trypsin is marked at pH 8.5 and negligible at pH 6.0 (7). Evidently then, if the enzyme preparation used contains such inhibitory substances, the more concentrated the enzyme solution the more the velocity of hydrolysis in the alkaline range will be slowed down compared to that at pH 6.0. This effect was in fact found to be marked with the glycerin extract if used direct or after partial dialysis but after the double dialysis described was too small to be detected. The presence of such effects can be tested for by diluting the enzyme with an inactivated portion of the same solution. The result of an experiment

with partially purified enzyme solution is shown in Fig. 2. The inactive enzyme markedly inhibits the reaction at pH 7 to 8 and has no effect at pH 6.

Extent of Hydrolysis.—It was found by Levene and Simms (8) that the hydrolysis of sarcosyl glycine, glycyl sarcosine, sarcosyl sarcosine, alanyl glycine, alanyl alanine, etc. did not go to completion but that there was an equilibrium between hydrolysis and anhydride formation. It is therefore necessary to consider the possibility that the position of the end-point depends on the pH. Experiments with excess enzyme, however, show that this is not the case but that the same final amount of hydrolysis takes place at all pH values. Complete hydrolysis occurs with all the peptides studied in this paper except glycyl d-l-leucine in which case the hydrolysis stops at 50 per cent, due either to anhydride formation or to the hydrolysis of only one isomer.

It was also found that fairly good monomolecular constants were obtained, except in the case of glycyl alanine which gave decreasing constants as the reaction

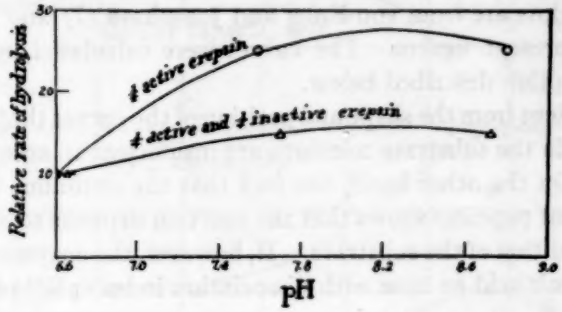

FIG. 2. Effect of concentration of erepsin on pH-activity curve. M/50 biuret base.

progressed. This is probably due to the fact noted by von Euler (7) that alanine has a more marked inhibitory effect than the other amino acids. The fact that the constants did not decrease with time show that under the conditions used, inactivation of the enzyme is negligible.

As a result of these experiments it may be said that the monomolecular constants for the hydrolysis, when run with purified enzyme solution and with dilute substrate, are a measure of the effect of the pH on the reaction and are independent of the substrate concentration below the value used and of the enzyme concentration provided this is the same in the experiments compared.

In running the experiments a series of peptide solutions were made up and titrated with NaOH so as to differ by about 0.5 pH from each other over the range from pH 4.5 to 10.0. They were then allowed to come to the desired temperature in the water bath and the enzyme solution added. Samples were then taken at 0.5, 1, 2, 4 hours etc. until the reaction was about 25 per cent complete. The

velocity constant was then calculated from the increase in formol titration by means of the formula

$$C = \frac{1}{t} \log \frac{A}{A - X} \quad (1)$$

where A is the maximum increase noted with excess enzyme. The largest velocity constant so obtained was taken as 100 and the other constants expressed in terms of this. Six to twelve experiments were run with each peptide and the final figures obtained by averaging the separate values found at the same pH in different experiments.

The results of the experiments are shown graphically in Fig. 3. The experimental points are represented by circles the size of which shows approximately the magnitude of the probable error of the mean. The solid dots are from von Euler and Josephson (7) and agree well with the present figures. The curves were calculated by means of the assumption described below.

It is evident from the shape and position of the curves that the known equilibria in the substrate solutions are insufficient to account for the results. On the other hand, the fact that the optimum varies with the different peptides shows that the reaction depends to some extent on the condition of the substrate. If, however, the enzyme is assumed to be a weak acid or base with dissociation index (pK′) of about 7.6 the curves may be predicted.

There are two possibilities either or both of which may lead to the pH optima found with the various substrates. Referring to the ionic species of the enzyme predominating above or below its dissociation index (pK′ = 7.6); or the ionic species of the substrates predominating above or below the indices given in Table II, it may be said that (I) the form of substrate predominating at low pH is acted on by the form of enzyme predominating at high pH, or that (II) the form of substrate predominating at high pH is acted on by the form of enzyme predominating at low pH, or that (III) both these reactions take place.

Other reactions must take place to a much smaller extent to agree with the observations.

The physical significance is clearer if the ionic species involved are considered, as summarized in Table I. In Table II it will be seen

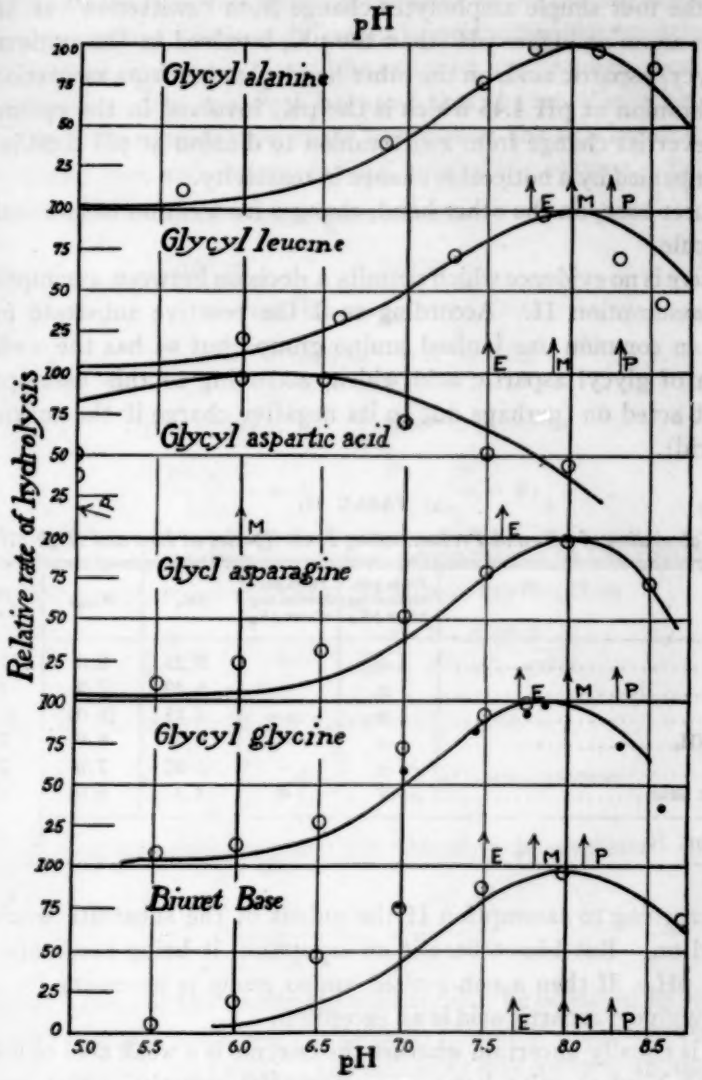

FIG. 3. Effect of pH on the rate of hydrolysis of various peptides.

○ observed.

— calculated.

The arrows indicate: pK_s of substrate, *P;* the observed maximum, *M;* and the corresponding value of pK_s (equation 7). Average $pK_s = 7.6$.

that the four simple ampholytes change from "zwitterion" at lower pH to anion at higher pH (than the pK_s involved in the optimum).

Glycyl aspartic acid, on the other hand, changes from zwitterion to zwitteranion at pH 4.45 which is the pK_s involved in the optimum. However its change from zwitteranion to dianion at pH 8.60 is not accompanied by a noticeable change in reactivity.

Biuret base, on the other hand, changes from cation to non-ionized molecule.

There is no evidence which permits a decision between assumption I and assumption II. According to I the reactive substrate forms have in common one ionized amino group—but so has the zwitteranion of glycyl aspartic acid which, according to this assumption, is not acted on (perhaps due to its negative charge if the enzyme is an acid).

TABLE II.

Calculation of pK_s and Predominating Ionic Species at Low and High pH.

	Form predominating below pK_s	Form predominating above pK_s	pK_s	H_{max}	pK_s calculated
GA	±	−	8.25	8.0	7.7
GL	±	−	8.29	7.9	7.5
GAp	±	±−	4.45	(6.0)	(7.6)
$GApNH_2$	±	−	8.3	8.0	7.7
GG	±	−	8.07	7.8	7.5
Biuret base	+	0	8.3	8.0	7.7
Mean					7.6

According to assumption II the anions of the substrate would be acted on. But biuret base is an exception, it being non-ionized at high pH. If then a non-ionized amino group is necessary for reactivity glycyl aspartic acid is an exception.

It is equally uncertain whether the enzyme is a weak acid or a weak base. Neutral salts have an exponential negative effect on the reactions which according to Brönsted indicates reaction between two oppositely charged ions. However, the activity of weak bases and ampholytes has been shown to be anomalous (14) and the significance of the effect of neutral salt is doubtful.

The formulation of the reaction is the same on the basis of any of the above assumptions, the only difference being the significance attached to the various constants. It will be assumed for purposes of calculation that the enzyme is a weak base and that the reaction is between the negative monovalent ion of the peptides or the non-ionized form of the biuret base and the non-ionized form of the enzyme. Let

(HA) = undissociated substrate

(HE) = " enzyme

B = total substrate = $A^- + (HA)$

D = " enzyme = $E^- + (HE)$

Then

$$(HA) \rightleftharpoons H^+ + A^- \qquad (HE) \rightleftharpoons H^+ + E^-$$

$$\frac{H^+ \times A^-}{(HA)} = K_a \qquad \frac{H^+ \times E^-}{(HE)} = K_a \tag{2}$$

and if the rate of reaction is assumed proportional to the product of the negative peptide ion and the non-ionized enzyme, then

$$\frac{-dB}{dt} = CA^-(HE) = \frac{C \cdot D \cdot K_a H^+ B}{(K_a + H^+)(K_a + H^+)} \tag{3}$$

and at constant substrate concentration and pH

$$\frac{C \cdot D \cdot K_a H^+}{(K_a + H^+)(K_a + H^+)} = \frac{1}{t} \log \frac{B_0}{B} = \text{velocity constant} \tag{4}$$

The observed velocity constant will therefore be expressed by the equation

$$C' = \frac{CK_a H^+}{(K_a + H^+)(K_a + H^+)} \tag{5}$$

and the position of the maximum velocity can be obtained by differentiating this expression with respect to C' and H. Setting dC/dH equal to 0 and solving for H_{max}, the following expression is obtained

$$H_{max} = \sqrt{K_a K_a} \tag{6}$$

or

$$pH_{max} = \frac{pK_a + pK_a}{2} \tag{7}$$

Since the values of the dissociation indices pK_s of the substrates are known it is now possible to solve for the value of the dissociation index of the enzyme pK_e. The results of this calculation are shown in Table II. The calculation gives a fairly constant value for pK_e of about 7.6 (*i.e.*, $K_e = 10^{-7.6}$). In order to obtain the entire curve this value of K_e may now be substituted in (5) and the equation solved for the value of the velocity constant C'. In order to compare these figures with those found experimentally it is necessary to express them as per cent of the maximum value. The maximum value found for C' is therefore taken as 100 and the other values calculated in proportion. These figures at the different pH were then used to plot the solid line shown in Fig. 3.

The calculation made above shows that the assumptions used are sufficient to account for the general shapes and position of the curve but involve several constants and are not very convincing in the absence of independent evidence. If the enzyme really has a dissociation index of about 7.6 it should be possible to show that some other property changes at that pH. It was found by Goulding, Borsook and Wasteneys (9) that the effect of the pH on the inactivation of pepsin agreed with the assumption that the enzyme was a weak electrolyte with a dissociation constant near pH 6. It seemed possible that a similar effect could be noted in the case of erepsin. The rate of inactivation of erepsin was therefore determined at various pH. The experiment was carried out in two ways, by determining the amount of enzyme in the solution after varying lengths of time by means of the rate of hydrolysis of glycyl glycine and also by following the decrease in the rate of hydrolysis of glycyl glycine with time at 35°C.

First Method.—A series of tubes containing the purified erepsin solution and 0.01 M glycine were adjusted to pH values about 0.5 pH units apart over the range of from pH 5 to 10 and put at 25°C. 1 cc. samples were removed at daily intervals and added to a stock solution of 0.1 M glycyl glycine pH 7.8. The rate of hydrolysis of the glycyl glycine and the velocity constant were then determined as usual and the concentration of enzyme assumed to be proportional to the velocity constant. The values obtained in this way for the concentration of enzyme were then plotted against time and the rate of inactivation of the enzyme calculated. The process was found to be approximately monomolecular. The velocity constants of these curves were then assumed proportional to the rate of inactiva-

tion of the enzyme. Inactivation proceeded slowly up to pH 6.5. It then became more rapid becoming constant above pH 9. The sample at pH 9 was half inactivated in about 3 days. This value was then taken as 100 and the other values expressed in proportion.

Second Method.—The hydrolysis of glycyl glycine under conditions such that the enzyme concentration remains unchanged is known to be monomolecular and the velocity constants are proportional to the enzyme concentration. If the experiment is arranged so that the enzyme is being inactivated the velocity constants when calculated for succeeding time intervals will drop and the values for each time interval may be assumed proportional to the average concentration of enzyme over that interval. This procedure furnishes a method therefore for determining the destruction of the enzyme in the presence of the substrate. In order to obtain significant results, however, it is necessary to arrange the experiment in such a way that the enzyme is inactivated before the substrate is hydrolyzed to a great extent (10). These conditions are fulfilled by carrying on the reaction with dilute enzyme and concentrated substrate and at a rather high temperature. A series of solutions 0.1 M glycyl glycine were therefore adjusted about 0.5 pH apart over the range of from 5 to 10 and placed at 35°C. The erepsin was added and the hydrolysis followed. A smooth curve was drawn through the figures obtained in this way and the monomolecular constants calculated for short time intervals. These were assumed to be proportional to the concentration of enzyme present at the corresponding time interval. The rate of inactivation of the enzyme could therefore be determined from them as described above.

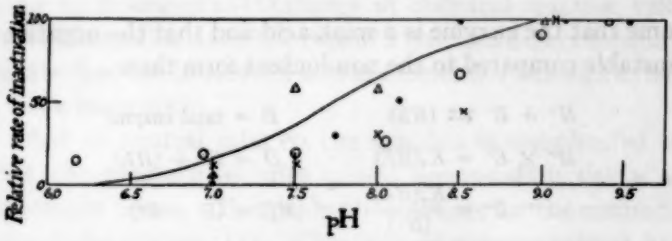

FIG. 4. Inactivation of erepsin at various pH.
— curve calculated from $pK_a = 7.7$.
○ inactivation at 35° + M/100 glycine.
△ " " 25° + M/50 glycyl glycine experiment (1).
● " " " " " " " " (2).
× " " " " " " " " (3).

The effect of the pH on the inactivation of erepsin as determined by these methods is shown graphically in Fig. 4. The solid line rep-

resents the calculated results assuming that the form of the enzyme present in acid solution is very stable compared to the form that is present in alkaline solution and that the value of the dissociation constant is $10^{-7.6}$.

As before it does not matter whether the enzyme is considered as a weak base or acid. The experimental points are irregular but lie in the region of the curve and a little to the alkaline side. The agreement is as good as can be expected for this type of experiment and furnishes independent evidence for the assumption made before that the enzyme had a dissociation curve near pH 7.6.

Calculation of the Rate of Inactivation.

Assume that the enzyme is a weak acid and that the negative ion is very unstable compared to the non-ionized form then

$$\begin{aligned} H^+ + E^- &\rightleftarrows (HE) & D &= \text{total enzyme} \\ H^+ \times E^- &= K_a(HE) & D &= E^- + (HE) \\ E^- &= \frac{K_a(HE)}{(H^+)} & HE &= (D - E^-) \end{aligned} \tag{8}$$

and

$$E^- = \frac{K_a D}{K_a + H^+} \tag{9}$$

If the rate of inactivation is proportional to the amount of the ionized form then at any constant pH

$$\frac{-dD}{dt} = C' E^- = \frac{C'K_aD}{K_a + H^+}$$

or (10)

$$\frac{C'K_a}{(K_a + H^+)} = \frac{1}{t} \log \frac{D_o}{D} = \text{velocity constant}$$

The reaction will therefore be monomolecular at any pH and the observed velocity constant will be given by the formula

$$C' = \frac{C'K_a}{(K_a + H^+)} \tag{11}$$

Substituting the value $10^{-7.6}$ for K_r and solving for the value of C″ at different values of H, a series of values are obtained which should be proportional to the observed inactivation constants. In order to compare them the maximum constant value found as the value of H becomes less, is taken as 100 and the values at the other pH expressed in proportion. The solid line in Fig. 4 has been plotted from these figures.

Effect of Neutral Salts.

It was stated above that the results agreed equally well with several assumptions in regard to the ionic species taking part in the reaction. According to Brönsted's (11) theory of chemical reaction velocities the effect of neutral salts on the rate of a reaction differs depending on whether the reaction is between ions of different or like sign or between an ion and a molecule.

The effect of neutral salts on the reaction is complicated by the fact that the presence of salts causes precipitation unless special precautions are taken. This probably accounts for the contradictory literature on the subject (12). The dialyzed glycerin extract becomes cloudy below pH 5 and this precipitation is prevented by the addition of neutral salts. Under these conditions neutral salts slightly increase the rate of hydrolysis. $CaCl_2$ on the other hand, causes a slight precipitate on the alkaline side of pH 7 and in this range retards the hydrolysis. These effects are presumably secondary and appear to be connected with the formation of the precipitate. It was found by Mr. Johnston that if the erepsin solution was dialyzed in the presence of 0.10 M $CaCl_2$ and then filtered a solution was obtained which remained clear over the range covered by the experiments. It was less active than the usual preparation. The effect of 0.10 M $CaCl_2$ on the hydrolysis of 0.01 M GG at various pH using this erepsin solution dialyzed in the presence of $CaCl_2$ is shown in Fig. 5. The salt inhibits at all reactions but the effect is much more marked on the alkaline side of pH 7 so that the presence of the salt causes a marked shift in the optimum. This result had been obtained by Abderhalden and Fodor (13).

The effect of increasing concentrations of NaCl and $CaCl_2$ on the rate of hydrolysis of glycyl glycine at pH 7.0 was then determined

with the erepsin prepared as described above. The result is given in Fig. 6 in which the relative rate of hydrolysis has been plotted against the log of the salt concentration. There is evidently a negative exponential salt effect which is Brönsted's prediction for a reaction between two ions of opposite charge. The only assumption in regard to the mechanism that predicts this is that the enzyme is a weak base and that the reaction is between the negative dipeptide ion and the positive enzyme ion. There is probably some doubt, however, as to the application of Brönsted's theory to catalytic reactions and the experiment can hardly be considered conclusive, especially since amines and ampholytes show anomalous changes in activity in the presence of neutral salts (14), particularly those of the type of $CaCl_2$.

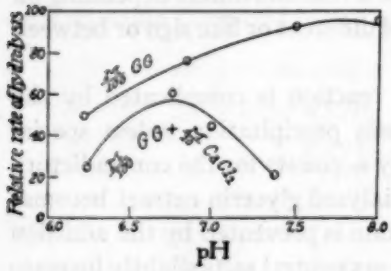

FIG. 5.

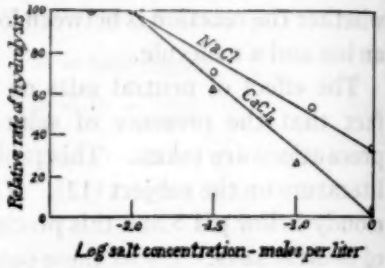

FIG. 6.

FIG. 5. Effect of M/10 $CaCl_2$ on rate of hydrolysis of M/100 glycyl glycine at various pH. Erepsin dialyzed in M/10 $CaCl_2$.

FIG. 6. Effect of $CaCl_2$ and NaCl on hydrolysis M/100 glycyl glycine pH 7.0.

Calculation of the Relative Rate of Hydrolysis of Different Substrates.

A number of workers have made determinations of the relative rate of hydrolysis of various peptides by erepsin, *i.e.*, the specificity of the enzyme. These determinations have, as a rule, been made at the same pH. It is evident, however, that if the mechanism assumed in this paper is correct, the determination of the relative rate of hydrolysis of different peptides is a difficult matter, except in the case where the pH-activity curves are the same for the different substrates. In general, however, it would be necessary to correct the observed rate for the fraction of the total enzyme and of the total substrate active at the pH used. The extent of this correction would vary

enormously depending on which of the various possible assumptions are used. In addition it must be shown that the relative rate of hydrolysis of the substrates compared is independent of the concentration. According to the experiments reported in the first part of the paper this is only true in very low concentrations. In view of these considerations it appears impossible, without further information, to make a significant comparison of the rates of hydrolysis of various peptides.

SUMMARY.

1. The rate of hydrolysis at different pH values of glycyl glycine, glycyl leucine, glycyl alanine, glycyl asparagine, glycyl aspartic acid and biuret base has been determined.

2. The pH-activity curves obtained in this way differ for the different substrates.

3. The curves can be satisfactorily predicted by the assumption that erepsin is a weak acid or base with a dissociation constant of $10^{-7.6}$ and that the reaction takes place between a particular ionic species of the enzyme and of the substrate. There are several possible arrangements which will predict the experimental results.

4. The rate of inactivation of erepsin at various pH values has been determined and found to agree with the assumption used above, that the enzyme is a weak acid or base with a dissociation constant of about $10^{-7.6}$.

5. It is pointed out that if the mechanism assumed is correct, the determination of a significant value for the relative rate of hydrolysis of various peptides is a very uncertain procedure.

Most of the experimental work reported in this paper was done by Mr. Frank F. Johnston.

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